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ERRATA

Vol. XXX, page 81. Along the ordinate of Fig. 2 (*a, b, c*):

for mg. CO₂/min. *read* mg. CO₂/0.25 min.

Vol. XXX, page 447. Left-hand column of table, lines 14 and 15:

for 5.0 α and 5.0 β *read* 2.5 α and 2.5 β

Vol. XXX, p. 236. Equation (2) should read

$$p_H = 12.87 - \log \left[\frac{[F]}{[F^-]} - 1 \right] - 0.19 [F] \quad \dots\dots(2).$$

Vol. XXX, p. 704

lines 3 and 25 *for* 6.0, 0.8 and 0.9 parts per million
read 0.06, 0.008 and 0.009 parts per million

line 5 *for* 70 parts per million *read* 0.06 part per million

Vol. XXX, p. 785. In Title of Paper

for William Cyras Sherman *read* William Cyrus Sherman

OBITUARY NOTICE.

HAROLD WARD DUDLEY.

1887-1935.

By the death of Harold Ward Dudley the Biochemical Society has lost a member to whom it will always be under a deep obligation. Besides the more ordinary service of acting on its Committee, he undertook the responsible work of secretary from 1922-4, and then joined Prof. Arthur Harden for six years in the arduous task of editing the *Biochemical Journal*. Many an author realises, and should be grateful for, the time and care which he gave, often out of the night hours, or during his week-ends, in order to improve their publications. The debt which Biochemical literature owes to him also includes his work for many years as assistant Editor of *British Chemical Abstracts*, which also occupied much of his time.

The eldest son of Florence and the late Rev. Joshua Dudley he was born at Derby and received his early education at Truro College and the King Edward VI Grammar School at Morpeth. Proceeding thence to Leeds University he specialised in Organic Chemistry under the late Prof. J. B. Cohen and obtained the M.Sc. degree in 1910 with a thesis on "The Relation of Position Isomerism to Optical Activity in certain Menthyl Esters". This initial association, and the subsequent award of an 1851 Science Research Studentship, proved to be crucial points in his career.

Cohen directed Dudley's attention, as he had already done that of others of his pupils who have since become famous, to the opportunities for research in the then new and growing science of biochemistry, and on his advice Dudley spent the next two years in Emil Fischer's laboratory in Berlin. To this great Master and to Dakin, with whom he worked later, he undoubtedly owed his wide knowledge of chemical technique and deep appreciation of the biological importance of the proteins, although his actual research in Berlin on the structure of purines, which gained him the degree of Ph.D. in 1912, was carried out under the immediate guidance of Prof. Wilhelm Traube.

On his return from Germany he was offered, and accepted, a research assistantship in Dr Herter's private laboratory in New York; there he had the rare good fortune to come under the influence of Dr H. D. Dakin, another of Cohen's old students and one of the most active pioneers in the field of protein and carbohydrate metabolism. He entered wholeheartedly into Dakin's researches, and the large number of important papers published under their joint names during 1913 and 1914 bears testimony to the fruitfulness of their collaboration. Moreover, the two men were of like temperament and a close friendship formed during this period was one which Dudley was to treasure throughout the remainder of his life.

The most noteworthy of their discoveries was perhaps that of the enzyme glyoxalase, which rapidly converts methylglyoxal at body temperature into lactic acid (*J. Biol. Chem.* 1913, **24**, 423). This was followed by a general method for the synthesis of alkylglyoxals (*J. Chem. Soc.* **105**, 2453), and the demonstration that these were also converted into the corresponding α -hydroxy-acids by glyoxalase enabled them to point out for the first time the importance of α -ketonic aldehydes in intermediary metabolism. They also extended Dakin's previous work on the

racemisation of proteins by alkali and showed that racemised caseinogen was not only unattacked by the usual proteolytic enzymes but was excreted unchanged by the animal body.

Returning to England in 1914 he was appointed lecturer in Biochemistry at Leeds; continuing work on the same lines he described a method for estimating the glyoxalase activity in blood (*Biochem. J.* 1915, 9, 97) and in collaboration with Dr H. E. Woodman showed by racemisation that the euglobulin and pseudoglobulin of cow's colostrum were structurally identical (*Biochem. J.* 1918, 12, 339) whereas the caseinogens from cow's and sheep's milk were not.

Before he had been long at Leeds, however, the growing importance of chemical warfare made it evident that his intelligence and knowledge would be of value in the service of his country. He accordingly joined the Army and on the formation of an anti-gas research department under the leadership of Lieut.-Col. E. H. Starling, began work at the Royal Army Medical College, Millbank, early in 1916. He was soon assisting the late Lieut.-Col. (then Lieut.) E. H. Harrison and other members of the team in the evolution and testing of the small box respirator and in many other ways applying his fertile mind to problems of chemical warfare. At Dudley's suggestion experiments were made in June 1916 which led to a recommendation to the Trench Warfare Committee that $\beta\beta'$ -dichloroethyl sulphide ("mustard gas") should be used as a gas-shell filling. The recommendation was rejected, but as is well known, the substance was used by the Germans from about June 1917, and onwards, with an initial success which we realised to our cost.

Having by 1918 acquired unique experience of the manufacture and testing of respirators, he was sent, with the rank of Major, in charge of a responsible Anti-gas mission to the United States. Having fulfilled these duties with characteristic care and thoroughness, in the course of which he made many and varied new friends, he was awarded the O.B.E.

On demobilisation from the Army he joined the staff of the Medical Research Committee (later Council) as their chief biochemist, a post that brought him for the first time into close contact with colleagues working in many different fields of medical research and permitted him to give full play to his matured intellect. Ever ready to learn, and to appreciate the difficulties of others, his interests naturally broadened and his subsequent work, which was done chiefly in collaboration with his colleagues, illustrates well his masterly skill in complex chemical analysis and the wide range of his activities. The close association which he formed with the director of the laboratories, Sir Henry Dale, was another fruitful friendship to which he owed a great deal and which he valued accordingly.

The first problem which he attacked was that of the chemical nature of the active constituents of the posterior lobe of the pituitary body. The results of the investigation fell short of his own expectations and high standard of achievement, but the work was nevertheless fundamental, besides being a monument of patience and technique. Having demonstrated clearly that the pressor and oxytocic actions were due to different substances (*J. Pharmacol.* 1919, 14, 295) and that the latter action was not due to histamine, and having succeeded in partially separating the two principles from one another, he attempted further to purify the oxytocic factor (Dale and Dudley, *J. Pharmacol.* 1921, 18, 27). At one stage he obtained a crystalline picrate of great potency, but further study showed this to be potassium creatinine picrate contaminated with small amounts of the active material which was thus shown to be immensely powerful (*J. Pharmacol.* 1923, 21, 103). It was characteristic of his stoical philosophy that he collected the picrate, labelled it "fiascone picrate" and kept it as a memento of labour lost.

Following the discovery of insulin by Banting and Best, the need of a standardised and economical method of manufacture which would place reasonable supplies at the service of hospitals and research workers was urgent, and after a visit to Toronto with Sir Henry Dale on behalf of the Medical Research Council to obtain first-hand information, Dudley devised the picrate method for its purification which gave a dry, stable product, having ten times the activity of the original crude insulin (*Biochem. J.* 1923, 17, 376). This was at once adopted by the insulin manufacturers in this country and is still in use to-day. His further work on insulin, which included the preparation of the first international standard, led him to suggest that the substance was a complex protein derivative, a conclusion which was to be amply confirmed later by other workers. News of his death reached the League of Nations Therapeutics Standards Committee at Geneva on October 3rd, while it was discussing the preparation of a fresh international standard of insulin, and was acknowledged by a fitting silence.

Meanwhile his interest had been partly diverted to a more chemical problem, and in collaboration with Dr O. Rosenheim and W. W. Starling he investigated the strong aliphatic base spermine, which was shown to be present in all the cellular tissues of the body and to have a structure that was afterwards confirmed by synthesis (*Biochem. J.* 1926, 20, 1082). While working up large quantities of material during this investigation they isolated a hitherto unknown base which they called spermidine, the properties of which suggested a structure related to, but simpler than, that of spermine. The correctness of this conjecture was also afterwards proved by synthesis (*Biochem. J.* 1927, 21, 97).

The importance of certain vasodilator constituents of the animal body with a histamine-like action for the normal functioning of the capillaries had long been emphasised by Sir Henry Dale. Dudley, in collaboration with Dr W. V. Thorpe, was able to identify the substance having this action with histamine itself which was isolated from several organs under conditions which precluded putrefactive changes. Next followed a brilliant research with Sir Henry Dale which led to the isolation of acetylcholine from horse and ox spleen (*J. Physiol.* 1929, 68, 97). Dudley's analytical skill was here shown at its best and his method of separating this unstable substance from choline and other substances present by means of their chloroplatinates bears the stamp of his genius (*Biochem. J.* 1929, 23, 1064). In 1930 his achievements were fittingly recognised in his election to the Fellowship of the Royal Society.

His last research, which was fortunately brought to a successful conclusion before his untimely death, dealt with the active principle of ergot. Until recently it has been assumed that the substances active towards the uterine muscle were the alkaloids ergotoxine and ergotamine, but in 1932 Dr Chassar Moir obtained direct evidence that aqueous extracts of ergot, free from these complex alkaloids, were still active when administered by the mouth. The active principle must therefore be different from any of those known hitherto, and Dudley undertook a chemical investigation of this unknown substance in association with the direct clinical observations of Dr Moir. The result was the isolation from ergot of a new, simple water-soluble alkaloid, ergometrine, which in minute doses has the characteristic activity of ergot. This discovery of ergometrine, which solves a longstanding problem of the pharmacology of ergot and its use in therapeutics, has aroused widespread interest as is shown by the fact that it has already been confirmed, in effect, in three other laboratories where research was independently in progress, and by the fact that the substance is now on the market for clinical use. The last paper on this subject from his pen was published on the day of his death (*Proc. Roy. Soc.* 1935, 118 B, 487).

OBITUARY NOTICE

In 1921 he married Mary Nettleship, who, after prolonged illness borne with outstanding courage, but which was a source of the deepest grief to him, died last April. She was a great help to him in his editorial work and members of the Biochemical Society will remember her fine singing at some of their larger functions.

Swift tragedy, which overtook him in his closing years, delivered its final blow when, on October 3rd, 1935, he died, three weeks after a serious and un-anticipated operation, being then nearly 48 years of age.

Although his manner was quiet and reserved, Dudley's was a personality of remarkable attractiveness, and no young man was held off by the gap of seniority from seeking his help. A keen fly-fisher and of musical and artistic tastes, he had a wide and varied circle of friends and was a well-known member of the Savage Club. Had he so wished, he could have had many more intimate and influential friends than he actually had, for he was a man of rare charm and fine character; in the doings of the distinguished his witty philosophy found much interest, but he never cultivated their friendship unless he saw in them more for admiration than for laughter; those whom he honoured will always treasure in their hearts the memory of his unselfish sympathy and affection, his unique placid wit and his patient repression of complaint under adversity.

A. C. C.

C. L. E.

I. INVESTIGATIONS IN ENZYMIC HISTOCHEMISTRY.

II. A MICRO-METHOD FOR THE DETERMINATION OF TRYPTIC ACTIVITY.

By LEOPOLD WEIL.

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(Received November 1st, 1935.)

THE development by Holter and Linderstrøm-Lang [1934] of methods and techniques for the micro-estimation of enzymes and other physiological substances in tissue sections may be said to mark the beginning of quantitative histological chemistry. In view of the applicability of these methods to biochemical problems, it becomes desirable to work out the technical details for the estimation of as many substances of physiological importance as possible.

The present paper describes a micro-method for the estimation of trypsin¹ using the apparatus developed by the above authors. The method is an adaptation of Sørensen's [1908] macro-method of formaldehyde titration of the carboxyl groups of proteins and their decomposition products. In preliminary studies it was found that the method of Foreman [1920] and Willstätter and Waldschmidt-Leitz [1921], namely, titration in alcoholic solution of the carboxyl groups liberated by tryptic action, is inapplicable because of precipitation of the protein substrate by the alcohol. A similar difficulty is encountered in the Linderstrøm-Lang acetone titration [1927]. Nephelometric measurement of tryptic activity, as described by Krijgsman [1934], is likewise unsuited to the micro-method because the instability of the turbidity may lead to considerable errors during a large series of experiments.

The formaldehyde titration method, on the other hand, was found to be very well suited to the present purpose. The addition of formaldehyde to the reaction mixture produces no precipitate, and at once prevents further enzyme action. The formaldehyde blocks the amino-groups liberated by the action of the trypsin, thus permitting the titration of an equivalent amount of carboxyl groups with alkali. As substrate, 4 % caseinogen solution at p_H 8.4 is used. Phenolphthalein is used as indicator, although thymol blue was also found to be satisfactory. Thymolphthalein cannot be used because of its insolubility in the aqueous formaldehyde solution. A standard for colour comparison was established by titration to a marked red colour, the latter being preserved by placing a soda-lime tube over the vessel.

The p_H optimum of the tryptic activity was found to extend over quite a wide range, in agreement with similar observations by Willstätter and Persiel [1925] and by Northrop [1922]. p_H 8.4 was selected as the optimum. In order that the formaldehyde titrations may be accurate, substances possessing dissociation constants within the titration range of p_H 6.8-9.5 must be avoided: phosphate buffer is, therefore, unsuitable for use in this titration. Borate buffer

¹ As is known, trypsin is a mixture of several proteolytic enzymes. The method described measures the tryptic activity of the whole complex.

reacts with the organic hydroxyl groups, and this may have a secondary influence on the enzyme. The veronal buffer at p_H 8.4, described by Michaelis [1930] is, therefore, to be preferred. This buffer, besides having a strong buffering power at this p_H , has the advantage of showing no reagent blank and, further, has no inhibiting effect upon tryptic activity.

Disturbances due to atmospheric CO_2 can be avoided during the reaction period by placing a soda-lime tube on the reaction vessel. Similar disturbances, which would affect the alkali titration, are prevented by employing the method developed by Linderstrøm-Lang *et al.* [1935], that is, the vessel in which the titration is carried out is closed by a bead, previously dipped in paraffin oil, the capillary end of the micro-burette passing through the hole in the bead and dipping into the solution being titrated. During the titration the mechanical stirring method described by Linderstrøm-Lang and Holter [1934] is used. A 0.05 *N* aqueous solution of tetramethylammonium hydroxide is employed to titrate the liberated carboxyl groups since, when sodium hydroxide is used, the carbonate is formed and crystallises out on the wall of the micro-burette, rendering the titration more difficult.

The method described, as shown by the experimental part, is suitable for histo-enzymic studies. It is reproducible to within $\pm 0.1 \mu l.$ of 0.05 *N* alkali.

EXPERIMENTAL.

Effect of formaldehyde concentration on accuracy of the titration. Increasing concentrations of formaldehyde were added to 0.1 *M* leucylglycine solution. The formaldehyde solutions were prepared by taking 1, 2, 4 and 8 ml. respectively of 40 % formaldehyde solution, adding 2 ml. of 0.1 % alcoholic phenolphthalein solution, titrating the solutions so obtained with 0.1 *N* sodium hydroxide to the first pink colour given by the indicator and diluting to 25 ml. with water. By means of a micro-pipette, as described by Linderstrøm-Lang and Holter [1934] and Glick [1935], 100 $\mu l.$ of the formaldehyde solutions thus prepared, containing 4, 8, 16 and 32 $\mu l.$ respectively of 40 % formaldehyde, were added to 7 $\mu l.$ of 0.1 *M* leucylglycine solution. It is advantageous to use freshly prepared aldehyde solutions. The titration of the carboxyl groups was performed by the method described by Linderstrøm-Lang *et al.* [1935] using 0.1 *N* aqueous solution of tetramethylammonium hydroxide. In order to carry all the titrations to the same end-point, as shown by the same indicator colour, the first determination was titrated to a marked red colour (above p_H 10), and this colour, preserved by placing a soda-lime tube on the reaction vessel, served as a standard. Using this method of protection, no change in the intensity of the standard colour was observed over a period of 5–6 hours. The results obtained, expressed in $\mu l.$ of 0.05 *N* tetramethylammonium hydroxide, are given in Table I. The value of the reagent blank (amounting to 0.2 $\mu l.$) has already been deducted from the titration results to obtain the figures given.

Table I. *Effect of formaldehyde concentration on accuracy of titration.*

Volume of 40 % formaldehyde in 100 $\mu l.$ solution $\mu l.$	Observed titra- tion (0.05 <i>N</i> alkali) $\mu l.$	Theoretical titration $\mu l.$
4	13.75	14.00
8	13.95	14.00
16	13.98	14.00
32	13.92	14.00

The solution containing 16 μ l. of 40 % formaldehyde in 100 μ l. solution, prepared as described above, was selected for further experiments since, as seen from Table I, further increase in the formaldehyde concentration causes no increase in the titration value. For each determination 100 μ l. of this solution were employed.

Micro-titration of known substances in presence of formaldehyde. To check the accuracy of the method, various concentrations of glycine, alanine and leucylglycine were titrated. The purities of these substances were checked by Kjeldahl determinations of total nitrogen. Solutions 0.1, 0.05, 0.033 and 0.02 *M* in concentration were made for each of the substances investigated, 7 μ l. of solution being taken for each determination. The free carboxyl groups were determined as described above. The results obtained (from which the reagent blanks have already been deducted) are shown in Table II.

Table II. *Micro-titration of known substances in presence of formaldehyde.*

	(μ l. 0.05 <i>N</i> alkali.)			
Substrate	0.1 <i>M</i>	0.05 <i>M</i>	0.033 <i>M</i>	0.02 <i>M</i>
Glycine	13.97	6.95	4.59	2.76
	13.97	6.92	4.61	2.82
	14.05	6.90	4.57	2.78
	13.97	6.99	4.62	2.78
	Average	13.99	6.94	4.60
Alanine	13.96	6.93	4.65	2.80
	13.97	6.93	4.67	2.82
	14.03	6.99	4.60	2.82
	13.99	6.97	4.63	2.80
	Average	13.98	6.95	4.63
Leucylglycine	13.96	6.92	4.60	2.74
	13.99	6.96	4.58	2.76
	13.90	6.96	4.54	2.76
	13.94	6.98	4.58	2.80
	Average	13.95	6.95	4.57
Calculated values	14.00	7.00	4.66	2.90

p_H optimum of tryptic activity. The trypsin solution used was prepared by taking 1 g. of dry trypsin preparation (Degoma S, Rohm and Haas Co.), suspending it in 10 ml. of water and allowing it to stand 1 day. To prevent bacterial infection a few drops of toluene were added. After 1 day the suspension was filtered and 0.1 ml. of the filtrate diluted to 25 ml. with water containing 30 % by volume of glycerol. To 7 μ l. portions of this enzyme solution 7 μ l. of 0.1 *M* veronal-hydrochloric acid buffer of *p_H* 6.4, 7.0, 8.0, 8.4, 9.0 and 9.5 were added together with 7 μ l. of a 4 % caseinogen solution of the same *p_H* as the buffer taken. "Hammarsten casein" was used and was adjusted to the required *p_H* by the addition of the necessary amount of *N* sodium hydroxide, the *p_H* being controlled by the hydrogen electrode.

The buffer and the enzyme solution were pipetted into the reaction vessel and the substrate carefully placed, as a drop, on the wall of the vessel and mixed with the reagents immediately before the experiments commenced. Simultaneously with each experiment a blank determination was done, identical with the determinations themselves except that the substrate was not mixed with the enzyme, but the reaction vessel was allowed to stand in a horizontal position (to avoid mixing of the solutions) under identical conditions. To avoid the effect of the CO₂ of the air on the experiments soda-lime tubes were placed on each

reaction vessel. After incubation for 30 min. at 37° the increase in the carboxyl groups was determined by the method of Linderstrøm-Lang *et al.* [1935].

The results are given in Table III, and are expressed in μ l. of 0.05 *N* tetramethylammonium hydroxide used. The corresponding blank determinations have already been subtracted.

Table III. p_H optimum of tryptic activity.

p_H	Increase in COOH groups (0.05 <i>N</i> alkali) μ l.
6.4	1.64
7.0	2.84
8.0	2.96
8.4	3.00
9.0	2.12
9.5	0.2

As the figures indicate, the p_H optimum of trypsin activity extends over a wide range, and for further experiments p_H 8.4 was selected.

Time course of tryptic activity as followed by the micro-method. 0.2 ml. of the enzyme extract (1 : 10), described above, was diluted to 25 ml. with water containing 30 % by volume of glycerol. To 7 μ l. of this enzyme solution 7 μ l. of 0.1 *M* veronal-hydrochloric acid buffer of p_H 8.4 and 7 μ l. of a 4 % caseinogen solution, also at p_H 8.4, were added. The increase in carboxyl groups was titrated, as previously described, using incubation times from 0 to 150 min. at 37°. The results are shown in Table IV, the figures given referring to μ l. of 0.05 *N* tetramethylammonium hydroxide required for the titration.

Table IV. *Time course of tryptic activity as followed by the micro-method.*

(μ l. 0.05 *N* alkali.)

Time min.	Control	Reaction mixture	Increase
0	2.80	2.84	0.0
7	2.76	4.46	1.70
15	2.76	6.07	3.31
22	2.84	7.44	4.60
30	2.80	8.55	5.75
45	2.78	9.85	7.07
60	2.80	10.79	7.99
90	2.84	11.41	8.57
120	2.80	11.95	9.15
150	2.82	12.36	9.54

As the figures show, the best condition for the reaction is that enzyme concentration, or that reaction time, which gives a carboxyl group increase requiring less than 7 μ l. of 0.05 *N* alkali.

Contact between enzyme and substrate. In histo-enzymic work using microtome tissue sections it is very important to show how far the enzymic activity is dependent upon the number of slices taken to obtain a given amount of tissue. It might be expected that by the use of several thin microtome slices, instead of one thick slice, to obtain a given amount of tissue, the contact between enzyme and substrate would be more complete. Fig. 1, taken from an unpublished study on tumour proteinase, shows that this factor, using the technique described, does not influence the results.

For this work a Walker No. 256 carcinoma was placed in a refrigerator for about 3 hours until frozen, after which a 2.5 mm. diameter pillar was prepared by

means of a cork-borer. The tumour pillar so obtained was placed on the freezing microtome and, from certain selected parts of it, two 25μ sections and four 12.5μ sections were taken immediately after each other for parallel trypsin determinations. Examinations of tumour tissues have shown that sections taken in this manner can be considered histologically identical.

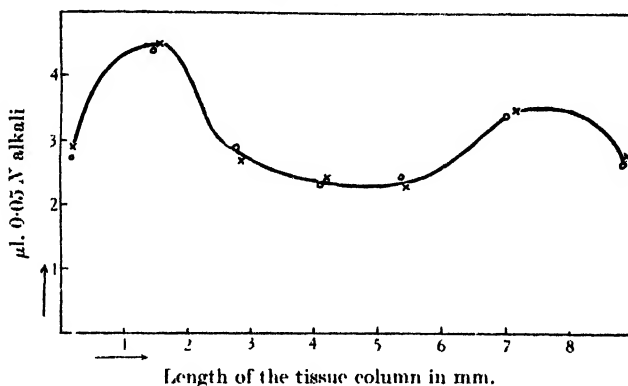


Fig. 1. Contact between enzyme and substrate.

○—○ Tryptic activity of two 25μ sections.

× — × Tryptic activity of four 12.5μ sections.

For the activation of the trypsin $7\mu\text{l.}$ of enterokinase¹ [Waldschmidt-Leitz, 1924] containing 30 volume % of glycerol were taken. The microtome sections were placed by means of a very fine glass rod in the drop of activator and allowed to stand for 1 hour at room temperature. After this period of activation, $7\mu\text{l.}$ of $0.1M$ veronal-hydrochloric acid buffer of p_{H} 8.4 were added together with $7\mu\text{l.}$ of a 4 % caseinogen solution also at p_{H} 8.4.

After 20 hours' incubation at 37° , using soda-lime tubes on the reaction vessels to prevent the diffusion of CO_2 from the atmosphere, the increase in the carboxyl groups was titrated as previously described.

As the curve demonstrates, no essential difference was observed between the results obtained by using two 25μ sections or four 12.5μ sections, thus showing that the present method is suitable for histo-enzymic studies.

The influence of extraction time upon the tryptic activity in microtome slices.

As Fig. 1 shows, the tryptic activity in the tumour tissue is independent of the thickness of the microtome slice over the range 12.5 – 25μ thickness. The second important question is whether, with increasing extraction time, a corresponding increase in the tryptic activity occurs. To study this problem slices from a carcinoma (Walker No. 256) were used, which were prepared from a frozen carcinoma pillar as described for Fig. 1.

Four 25μ sections were obtained from a selected portion of the tumour pillar, two of them being placed in $7\mu\text{l.}$ of 30 % (volume) aqueous glycerol solution and two, to give full activity, being placed in $7\mu\text{l.}$ enterokinase solution containing 30 % by volume of glycerol. These solutions were allowed to stand for 1 hour. Exact duplicates were made, using four 25μ slices from the adjacent portion of the tumour pillar, and these were allowed to stand for 6 hours. After addition of

¹ The enterokinase solution alone did not affect either the caseinogen or the leucylglycine solutions during the reaction period.

7 μ l. of 0.1 *M* veronal-hydrochloric acid buffer and 7 μ l. of 4 % caseinogen solution, the solutions were allowed to stand 20 hours at 37°, and the increase of carboxyl groups was measured as described above.

As Fig. 2 shows, practically no difference was observed in the tryptic activity whether the extraction time was 1 or 6 hours. Preliminary experiments indicate

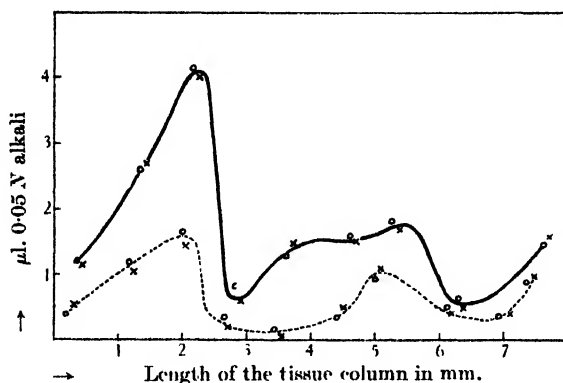


Fig. 2. The influence of extraction time upon the tryptic activity in microtome slices.

- Full activity after 1 hour's extraction time.
- ×—× Full activity after 6 hours' extraction time.
- Initial activity after 1 hour's extraction time.
- ×---× Initial activity after 6 hours' extraction time.

that only a certain part of the tryptic activity is thus extracted, the remainder of the activity being connected with the tissue. This phenomenon may be compared with the lyo- and desmo-enzymes studies by Willstätter and Rohdewald [1932] and with a similar observation on pepsin by Holter and Linderstrøm-Lang [1934].

SUMMARY.

The present paper describes a micro-method for the determination of tryptic activity in tissue sections. The reproducibility of the method is ± 0.00007 mg. amino-nitrogen. The method is suitable for histo-enzymic studies.

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II. OBSERVATIONS ON THE EXCRETION OF VITAMIN C IN HUMAN URINE.

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SINCE the chemical identification of vitamin C, and the development of a chemical method for its estimation, the excretion of this vitamin in human urine has attracted attention [Harris *et al.*, 1933; Johnson and Zilva, 1934; Harris and Ray, 1935]. The constant daily loss of 30–33 mg. of ascorbic acid in the urine of normal individuals as estimated by Harris and his colleagues did not appear to us quite in accord with accepted notions about the function and requirements of vitamins. It was conceivable that a biological fluid of such complex composition as urine should contain besides ascorbic acid, substances capable of reducing the indicator used in this estimation. Our doubts were further strengthened when a preliminary investigation, covering a period of a week, showed only half as much vitamin C in the daily food of an individual who was found to be excreting in his urine an amount similar to the estimate of Harris and his co-workers. We were, therefore, led to study the excretion of ascorbic acid in the urine and to explore the possibility of the presence of other reducing substances.

The daily output of the reducing substance or substances in human urine under normal conditions was estimated by the usual titration technique for a number of individuals. The results are expressed as mg. of ascorbic acid and are shown in Table I. For different persons the total daily excretion was found to be variable, but for the same person the day to day excretion was strikingly uniform. The level of output stood between 23 and 35 mg. in terms of ascorbic acid. These findings are in conformity with the results of Harris and his colleagues.

Table I. *The reducing capacity of normal urine.*

(Expressed as mg. ascorbic acid.)

Name of the subject	Days	Total volume of urine passed (ml.)	Conc. of reducing substance (mg./100 ml.)	Total reducing substance excreted (mg.)
B. A.	1	512	4.62	23.67
	2	594	4.26	25.39
	3	649	3.91	25.28
S. C. N.	1	549	4.41	24.11
	2	710	3.31	23.50
D. N. M.	1	980	3.47	34.03
	2	903	3.83	34.65
H. E. C. W.	1	641	4.64	29.77
H. D. A.	1	892	3.67	32.78
	2	1510	2.34	35.36
M. D. A.	1	1216	2.13	26.00
	2	1736	1.41	24.53

One of the subjects (B. A.) was then given large daily doses of ascorbic acid in the form of fresh juice of sweet limes and the reducing capacity of the urine was examined at short intervals. The results (Table II) mainly confirm the observations of Harris and his colleagues and of Johnson and Zilva [1934].

Table II. *The reducing capacity of urine after ingestion of large doses of ascorbic acid.*

(Expressed as mg. ascorbic acid.)

Days	Dose of ascorbic acid (mg.)	Total reducing substance excreted in 24 hours (mg.)	Days	Dose of ascorbic acid (mg.)	Total reducing substance excreted in 24 hours (mg.)
1	0	19.65	8	0	33.03
2	200	24.34	9	0	35.95
3	460	41.37	10	0	27.39
4	660	351.17	11	50	72.16
5	0	74.24	12	50	50.54
6	0	27.66	13	50	48.68
7	0	30.25			

The reducing capacity of urine did not increase until the so-called "saturation point" for the tissues was reached after repeated or increased doses of ascorbic acid. The excretion of ascorbic acid was then quite rapid, beginning within 2 hours after the dose, attaining a maximum rate of output after 4-7 hours and then slowly returning to the normal level at the end of 40 hours. Even after the "saturation point", the total excretion by no means approximates to the total intake. The reducing power of urine accounts for only about 50 % of the total quantity taken. A week after attaining the "saturation point", a small dose of 50 mg. ascorbic acid was repeated. The response as increased excretion of reducing substances was immediate, showing that the tissues of the subject were still "saturated".

The excretion of reducing substances was now studied under different dietary conditions. Three different diets were first used.

I. A low vitamin C diet consisting of a preponderatingly large proportion of cereal and bread, and containing only small amounts of meat, milk, eggs and vegetables. This was the usual diet of the subject.

II. Low protein diet. A relatively large proportion of vegetables was included with moderate amounts of bread and cereal. Meat, eggs and milk were altogether eliminated from the diet. This diet contained nearly three times as much vitamin C as diet I.

III. High protein diet. This consisted largely of meat and fish together with commercial meat extracts (Lemco), and some bread.

The examination of the urine was begun after the subject had been on the diet for 3 days. The results are summarised in Table III.

During the course of each diet the daily output of the reducing substance was quite constant. On diet I it was about 25 mg. On diet II which had three times more vitamin C than diet I, the reducing capacity of the urine represented only 19-20 mg. ascorbic acid. On the high meat diet the daily excretion increased to 45-65 mg.

Since diet III included beef extracts which generally cause a greater excretion of purines, we suspected that substances of a purine nature might possibly be responsible for the increased reducing action of urine, although the possibility

Table III. *The reducing capacity of urine excreted under different dietary conditions.*

(Expressed as mg. ascorbic acid.)

Diet	Days	Vol. of urine passed (ml.)	Conc. of reducing substance (mg./100 ml.)	Total reducing substance excreted (mg.)	Total nitrogen (g.)	Approx. ascorbic acid ingested in food (mg.)
I	1	512	4.62	23.67	—	13.2
	2	479	4.38	21.00	9.05	13.4
	3	594	4.27	25.38	10.03	11.0
	4	655	3.90	25.53	9.94	14.1
	5	592	4.23	25.05	8.58	52.3
	6	649	3.89	25.26	9.36	—
	7	756	3.88	29.36	9.98	—
II	1	814	2.49	20.29	9.00	44.5
	2	787	2.50	19.71	6.87	113.4*
	3	643	3.08	19.83	6.92	40.6
	4	599	3.08	18.48	6.08	43.2
	5	544	3.61	19.65	5.87	—
III	1	670	7.27	48.71	10.84	19.0
	2	614	7.29	44.79	11.05	18.5
	3	572	11.29	64.62	10.70	18.5
	4	642	9.63	61.68	9.56	20.8
	5	620	7.99	49.57	10.55	18.5
	6	911	6.31	57.49	11.93	20.0
	7	675	6.62	44.73	10.36	20.0

* These high figures are due to the ingestion of a vitamin C-rich fruit.

that the metabolism of proteins by themselves may demand increased metabolism of vitamin C, could not be ignored.

A further extended study of the urine excreted during different diets was continued. In addition to the three diets mentioned above a fourth diet was also included.

IV. Purine-free, high protein diet, containing larger quantities of eggs and milk with small amounts of bread, vegetables and fruit. The results are shown in Table IV.

The results are essentially similar to those of previous experiments. The reducing value of the urine is low on diets I, II, and IV. It increases with a change to the high meat diet III. There is no increase with diet IV.

Table IV. *Analyses of urine excreted during different diets.*

(Reducing substances expressed as mg. ascorbic acid excreted in 24 hours.)

No. of days	Diet	Total volume of urine (ml.)	Total reducing substances (mg.)	No. of days	Diet	Total volume of urine (ml.)	Total reducing substances (mg.)
1	I	659	40.50	13	I	635	37.50
2	I	518	18.87	14	I	710	33.84
3	I	524	18.30	15	I	636	25.46
4	II	608	17.64	16	I	637	33.33
5	II	445	16.83	17	I	879	28.60
6	II	485	16.10	18	I	655	25.65
7	IV	720	15.46	19	I	585	21.20
8	IV	336	14.00	20	I	771	27.10
9	IV	512	16.25	21	III	1331	62.10
10	III	721	151.15	22	III	801	66.37
11	III	802	88.21	23	III	806	81.34
12	III	804	266.37	24	III	795	64.34

The exceedingly high values of the reducing substance obtained on the 10th, 11th, and 12th days are not altogether due to a high meat diet. From the 8th to 11th days the subject took one "langra" mango every day. The very high values obtained aroused our suspicions about the mango and on examination it was found that this particular variety of the mango was exceedingly rich in vitamin C. The subject went over to diet I again and changed to diet III on the 21st day. The usual increase in the reducing capacity of urine was found to take place.

Biological test of urine.

It was decided to test the antiscorbutic value of urine excreted on diet III. A group of eight guinea pigs was put on the usual scorbutic diet. Four animals received every day two different doses of fresh urine equivalent in reducing capacity to 0.5 and 1.0 mg. ascorbic acid. The remaining four animals acted as controls. The results of the tests are summarised in Table V.

Table V. *The antiscorbutic value of urine.*

No. of animal	Dose of urine (ml.)	Ascorbic acid equivalent of the dose (mg.)	Total time of test (days)	Total change in weight (g.)	Average growth per week (g.)	Remarks
1	3.2-8.0	0.5	32	- 40	- 8.7	Died, p.m. scurvy
2	3.2-8.0	0.5	37	- 195	- 36.8	"
3	6.4-16.0	1.0	29	- 135	- 32.6	"
4	6.4-16.0	1.0	27	- 119	- 30.8	"
5	0	0	38	- 165	30.3	"
6	0	0	38	- 210	- 38.6	"
7	3 ml. of lime juice	1.0	38	- 10	- 1.8	Healthy, killed p.m. no scurvy
8	"	1.0	42	- 60	- 10.0	Healthy

All the four animals receiving a dose of urine died between the 27th and 37th days of the experiment, and the *post mortem* examination showed evidence of scurvy. The negative controls died of scurvy after somewhat longer time as compared with the animals receiving urine, which indicated a possible additional toxic effect of urine. The controls which were fed 3 ml. of lime juice remained quite healthy.

These experiments may appear to support the view that probably the whole of the reducing action of urine excreted during high protein diet, is not due to ascorbic acid. The toxicity of urine, however, is a factor which must be taken into consideration in these experiments. Even after a week's feeding of urine the animals began rapidly to lose weight and looked ill. It is more than likely that under these conditions the absorption and metabolism of ascorbic acid of the urine is interfered with.

The reducing substances in the urine were next studied from the point of view of their stability to heat and their reaction with lead acetate. A striking similarity in behaviour was revealed between the reducing substances of the urine and pure ascorbic acid under both these treatments. The results of one of these experiments are summarised in Table VI.

The rates of destruction of natural reducing substances in urine and of pure ascorbic acid in solutions of urine are evidently very close.

The experiments described above show that there is a definite rise in the reducing capacity of urine when the subject changes from a low or a moderate

Table VI. *The relative heat-stability of the reducing substances in urine and of ascorbic acid.*

(Reducing value expressed as mg. ascorbic acid.)

	Treatment	Reducing substance mg./100 ml.	% loss of reducing substance
I. Urine acidified with acetic acid Urine of diet III	Nil	8.88	0
	Boiled 5 min.	8.23	7.32
	Boiled 15 min.	6.35	28.48
	Boiled $\frac{1}{2}$ hour	5.55	37.49
II. A mixture of urine and pure ascorbic acid acidified with acetic acid	Nil	22.20	0
	Boiled 5 min.	20.40	8.60
	Boiled 15 min.	16.68	23.94
	Boiled 30 min.	14.28	35.64

meat diet to a high meat one. The quantity of meat consumed during high meat diet was 1-2 lb. per day supplemented with 0.5-1.0 oz. of lemco. The increase in the total daily excretion of the reducing substance is as much as two to three times the ordinary output.

The evidence taken as a whole appears to favour the view that the increased reducing action of urine is to a large extent due to increased excretion of ascorbic acid. The biological experiments on the antiscorbutic value of urine are not conclusive on account of the complicating effect of the toxicity of the urine. On the other hand the general behaviour of the reducing substance resembles very much that of ascorbic acid.

If a high meat diet favours a rapid elimination of ascorbic acid by way of urine, we have here a possible explanation of the high incidence of scurvy among sailors during the earlier days. Their diet was chiefly salted meat and ship biscuits, which would quickly deplete their tissues of their stores of vitamin C.

SUMMARY.

The reducing capacity of human urine excreted under different dietary conditions has been studied. There is a considerable amount of increase in the reducing action of urine on high meat diets without any increase in the vitamin C content of the food.

The possibility that urine under these conditions may contain besides ascorbic acid other substances capable of reducing dichlorophenolindophenol in acid solution has been examined. The evidence presented is generally in favour of the view that even on high meat diets the greater part of the reducing substance of urine is ascorbic acid. It appears that high meat diets favour the excretion of vitamin C by way of urine.

I wish to express my indebtedness to Prof. H. E. C. Wilson for his constant interest and help during the course of this investigation, and to Lt.-Col. A. D. Stewart, Director, All India Institute of Hygiene and Public Health, Calcutta, for his valuable criticism and permission to publish these results.

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III. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLVII. THE PRODUCTION OF POLYSACCHARIDES BY *PENICILLIUM LUTEUM* ZUKAL.

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It was shown by Raistrick and Rintoul [1931] that *Penicillium luteum* Zukal, when grown on Czapek-Dox medium containing glucose as the sole source of carbon, produced as the main metabolic product large yields of luteic acid, a malonyl-polyglucose, the products of acid hydrolysis of which consisted only of glucose and malonic acid. Alkaline hydrolysis of luteic acid gave rise to a neutral polysaccharide, luteose, by fission of the malonic acid groups. Later, Birkinshaw and Raistrick [1933] showed that luteic acid was elaborated by the same organism even if the glucose of the medium were replaced by any one of the following compounds as sole source of carbon: fructose, galactose, mannose, xylose, arabinose and glycerol.

During an investigation of the molecular constitution of luteose [Anderson *et al.*, unpublished] it was found that in the total crude material there was mixed with the luteose 8–10% of another polysaccharide which yielded trimethyl-mannose after methylation and hydrolysis. The present work was undertaken in an attempt to trace the course of formation of this polymannose.

Flasks of Czapek-Dox medium containing 5% of glucose as the only carbon source were sown with a spore suspension of *P. luteum* Zukal, incubated at 24° and examined for polysaccharide content at intervals. Half the flasks were worked up when approximately 3.5% of glucose still remained (33 days) and the other half when the glucose had almost entirely disappeared (108 days). The product from each batch was separately submitted to an exhaustive fractionation from aqueous solution by alcohol. By this means the polysaccharide from the younger cultures yielded 8 fractions having different optical rotations and that from the older cultures 7 fractions.

The product from young cultures yielded large quantities of material having a specific rotation $[\alpha]_{5461} -30^\circ$ to -40° (luteic acid has $[\alpha]_{5461}^{21^\circ} -37.7^\circ$) together with smaller amounts of material having $[\alpha]_{5461} -5^\circ$ to -15° , whilst that from old cultures yielded, in addition to the above products, considerable quantities of material having a high specific rotation, $[\alpha]_{5461} -80^\circ$. Examination of each of these fractions for malonic acid, hexoses and pentoses showed that glucose, mannose and malonic acid were invariably present whatever the age of the culture; galactose was found in some of the fractions, particularly those from old cultures; fructose was found in small amount in some of the fractions, whilst pentoses were invariably absent. The results show that the main product is always a malonyl-polyglucose, that a mannose polysaccharide is found largely in the early stages of growth and that it tends to be replaced by a galactose polysaccharide in older cultures. The polysaccharides produced by young

cultures are hydrolysed less readily by acid than those found in old metabolism solutions.

It seems possible that the production by *P. luteum* of a series of polysaccharides containing different sugars may be closely linked with the formation of luteic acid from a variety of sugars reported by Birkinshaw and Raistrick [1933], both processes involving an intermediate product obtainable from all sugars and capable of being rebuilt into one or more carbohydrate units according to conditions. Support for this view is afforded by the observations of Coyne and Raistrick [1931] on the production of mannitol from hexoses and pentoses by a white species of *Aspergillus*, by the production of cellulose from glucose, sucrose, fructose, mannitol, glycerol and glyceraldehyde by *Bact. xylinum* [Hibbert and Barsha, 1931; Tarr and Hibbert, 1931] and confirmed by the work of Khouvine *et al.* [1932] and of Khouvine [1933], which showed that *Bact. xylinum* was capable of elaborating cellulose from glycerol, sorbitol, mannitol and α -glucoheptitol. A similar conversion of glucose into polysaccharides composed of other carbohydrate units was reported by Clutterbuck *et al.* [1934] who isolated a polymannose and a polygalactose as a result of the action of *P. Charlesii* G. Smith on glucose. Analogous also are the cases of the production of a polysaccharide containing galactose, glucose and idose or altrose found by Haworth *et al.* [1932; 1935] as a result of the cultivation of *P. varians* G. Smith on Czapek-Dox glucose medium. *P. rugulosum* Thom also produces a galactose polysaccharide when grown on Czapek-Dox medium containing glucose as sole source of carbon [Anderson and Raistrick, unpublished]. In this connection may also be cited the report by Dox and Neidig [1914] of the isolation of mycogalactan by extraction of mycelia of *Aspergillus niger* Van Tieghem grown on Raulin's medium.

EXPERIMENTAL.

50 one-litre conical flasks, each containing 350 ml. of Czapek-Dox medium of the following composition, were made up: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 litre. After sterilisation each flask was sown with a spore suspension of *Penicillium luteum* Zukal (L.S.H.T.M. Catalogue No. Ad 30). This is the same strain as was used by Raistrick and Rintoul [1931], prepared from 17 Czapek-Dox glucose agar slopes 10 days' old. The flasks were incubated at 24° and single flasks were tested at intervals with the results given in Table I.

Table I.

Incubation period in days	α_{5790}	% (glucose by		Titration ml. 0.1 N NaOH/ 100 ml	Yield of crude polysaccharide g. per flask
		Polarimeter	Shaffer- Hartmann		
25	+ 4.14°	3.78	3.76	12.0	1.28
31	+ 3.97°	3.64	3.60	12.0	0.94
46	+ 2.35°	2.15	2.51	10.0	1.40
59	+ 1.76°	1.60	1.86	7.0	1.42
108	- 0.22°	—	0.04	2.5	1.75

(A) Examination of polysaccharides produced after short incubation period.

After 33 days' incubation the contents of 23 flasks were filtered through a "Metafilter" and concentrated *in vacuo* to 1 litre. 15 ml. of concentrated HCl led and the polysaccharides fractionally precipitated with alcohol. The

precipitates obtained with different concentrations of alcohol were filtered, washed with alcohol and ether, dried and their optical rotations determined. The different fractions obtained were grouped according to rotations and were refractionated until a final series of 8 fractions was obtained (Fractions I to VIII—Table II). Each of these fractions was then examined as follows:

(i) Determination of equivalent before and after alkaline hydrolysis by titration with $N/10$ NaOH, treatment with excess of $N/10$ NaOH and back-titration.

(ii) Determination of specific rotation.

(iii) Hydrolysis by N , $2N$, or $3N$ H_2SO_4 at 85° followed polarimetrically.

(iv) Determination of the amount of reducing sugar produced on hydrolysis with H_2SO_4 (this determination was only carried out for fractions weighing 1 g. or more).

(v) Extraction of the hydrolysate from IV with ether and characterisation of the acid extracted by ether as malonic acid by sublimation, melting-point and acid equivalent.

(vi) Neutralisation of the ether-extracted hydrolysate from V with $BaCO_3$. The H_2SO_4 -free hydrolysate was concentrated *in vacuo* and tested for:

(a) Mannose by formation of mannosephenylhydrazone on treatment with phenylhydrazine in the cold.

(b) Glucose by formation of glucosazone after removal of mannose as the phenylhydrazone.

(c) Fructose by Bredereck's reaction [1931] (ammonium molybdate in HNO_3).

(d) Galactose by formation of mucic acid on oxidation with HNO_3 .

(e) Pentoses by Bial's reaction (orcinol + $FeCl_3$ in concentrated HCl).

In some cases it was possible to isolate glucose and galactose as such. The results obtained are summarised in Table II, Fractions I–VIII inclusive.

(B) *Examination of polysaccharides produced after long incubation period.*

The contents of the remaining 23 flasks were filtered after 108 days' incubation and concentrated to 350 ml. (this solution was considerably less viscous than that from the 33-day batch). 7.5 ml. of concentrated HCl were added and the

Table II.

Fraction	Weight g.	Equivalent		Rotation $[\alpha]_{5461}$		% reducing sugar by	
		Before hydrolysis	After hydrolysis	Initial	Final	Shaffer- Hartmann	Alkaline I
I insol. in cold water	1.06	996	413	—	+13°	—	—
II	7.29	864	335	−40.0°	+64.5°	82	93
III	8.35	1065	270	−30.0°	+73.5°	—	75
IV	4.59	2240	377	−15.0°	+60.4°	—	62
V	0.23	2500	300	−26.5°	+61.0°	—	—
VI	1.69	Neutral		−21.6°	+32.5°	—	—
VII	1.88	705	300	−32.7°	+54.5°	40	45
VIII	0.41	Neutral		−5.2°	+5.5°	—	—
IX insol. in cold water	11.95	8930	1880	−38.7°	+63.5°	84	93
X	0.24	6900	1530	−83.2°	+92.5°	—	—
XI	2.23	10000	1100	−80.5°	+86.0°	78	85
XII	3.11	15700	2245	−51.0°	+90.3°	70	86
XIII	4.03	13230	1235	−42.3°	+80.1°	75	90
XIV	0.55	6960	1245	−42.3°	+72.0°	—	—
XV	2.06	528	330	−11.6°	+52.4°	37	43

polysaccharides were fractionally precipitated with alcohol, refractionated and examined in the same way as is described under Section A. The results obtained are summarised in Table II, Fractions IX to XV inclusive.

Discussion of results.

In all the fractions, whether from the young or old cultures, glucose in large amounts and malonic acid in smaller amounts were present and pentoses absent. Mannose was present in appreciable amount in Fractions II, V, VI, VII, X, XIV and XV and in traces in all the remaining fractions. Galactose was actually isolated from Fractions XII and XIII, was detected in traces in Fractions I, II, V, VIII, XI and XIV, and was absent from the remaining fractions. Fructose was present in small amounts in Fractions II, III, XI and XIII, in traces in Fractions IV, IX, XII and XV, and was absent from the remaining fractions.

It is evident that young cultures up to 33 days produce a preponderance of material with a low laevorotation, that is $[\alpha]_{D_{441}} - 40^\circ$ or below, and none above $[\alpha]_{D_{441}} - 40^\circ$. On the other hand, the old cultures (108 days) produce a considerable amount of material having a much higher specific rotation $[\alpha]_{D_{441}} - 80^\circ$, but little material having a small laevorotation. It is also seen that the older cultures produce considerably more polysaccharide material insoluble in cold water than do the younger cultures. Further, much of the polysaccharide produced by old cultures is much more readily hydrolysed than that from the young cultures. A notable difference between polysaccharides from old cultures and those from young cultures is the marked increase in the acid equivalent with age, both before and after alkaline hydrolysis.

It thus appears that whilst luteic acid, defined as a malonyl-polyglucose, is the principal metabolic product of *P. luteum* Zukal at all stages of growth, it is accompanied by a polymannose in the early stages of growth, and that this polymannose tends to be replaced by a polygalactose with increasing age of the culture.

SUMMARY.

Penicillium luteum Zukal, when grown on a Czapek-Dox medium containing glucose as the sole source of carbon, produces as the main metabolic product luteic acid, a malonyl-polyglucose. In addition however there are formed much smaller amounts of other laevorotatory polysaccharides, built up of mannose, galactose or fructose units in proportions varying with the age of the culture.

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IV. FAT METABOLISM IN FISHES.

VIII. CHANGES IN THE FAT OF RIPENING SALMON EGGS.

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In a previous paper [Lovern, 1934, 2] it has been shown that the fat of ripe salmon eggs is different in type from salmon body fat. Apart from higher average unsaturation of the C_{20} and C_{22} acid groups, the content of C_{18} acids is distinctly higher. The egg fat indeed suggests a fat of the fresh-water type, with the specific peculiarities of the salmon superimposed upon it. It was further shown that whilst this egg fat must have been deposited selectively, the selection was not exercised by any specific withdrawal from the depots. The work now to be described is a closer study of the mechanism of this selective deposition of egg fat.

EXPERIMENTAL.

Samples of salmon ova were collected in various stages of maturity. The least developed were obtained from "fresh run" fish in February and March. Intermediate samples were obtained from fish taken up river in May, July and September. Fully ripe ova were obtained from fish actually spawning in the Tweed during November of 1934. In order of increasing development these will be referred to as samples Nos. 1 to 5. Sample No. 1 represented about 100 fish, the others considerably fewer. No. 3 was from one fish only. Apart from No. 3, however, sufficient fat was available in each case for analysis by full-scale methods. The fat contents of the ova were found to be irregular, but of the same order throughout. They ranged from 7 to 15%. The fats were freed from phosphatides, and their characteristics are given in Table I. The most significant variation is the steady fall in iodine value with ripening.

Table I. *Particulars of salmon ova fats.*

No.	Iodine value	Sap. equivalent	Unsaponifiable %
1	206.1	315.9	8.8
2	198.2	320.6	7.4
3	194.3	327.1	9.0
4	174.8	315.9	6.3
5	176.1	311.9	7.2

The compositions of the mixed fatty acids (as wt. %) are given in Table II. In the case of No. 3 which had to be examined on a semimicro-scale, a series of accidents occurred. These rendered it impossible to obtain a reliable complete analysis for this fat, but it was possible to calculate the content of C_{18} unsaturated acids and the degree of average unsaturation of these. In Table II are included

for comparison the results on the ripe ova fat from "baggots"¹ recorded previously [Lovern, 1934, 2].

Table II. *Fatty acid compositions (Wt. %).*

No.	Saturated				Unsaturated				
	C ₁₄	C ₁₆	C ₁₈	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	
1	3.1	16.0	0.5	0.1	12.6 (-2.0H)	23.7 (-4.0H)	27.2 (-8.0H)	16.8 (-10.4H)	
2	2.9	13.4	0.7	0.4	10.4 (-2.0H)	25.3 (-3.2H)	28.7 (-7.2H)	18.1 (-11.4H)	
3	—	—	—	—	—	28.0 (-3.1H)	—	—	
4	1.8	13.0	2.0	—	9.9 (-2.0H)	36.7 (-2.9H)	21.3 (-7.6H)	15.3 (-11.1H)	
5	2.3	12.9	2.2	—	9.6 (-2.0H)	34.8 (-2.7H)	23.2 (-7.6H)	15.0 (-11.2H)	
Baggot	2.3	11.2	1.9	—	12.3 (-2.0H)	34.4 (-2.7H)	21.7 (-7.2H)	16.2 (-9.5H)	

DISCUSSION.

Table II reveals several points of interest. (a) It will be seen that although "baggots" are abnormal fish as regards spawning, yet the egg fat is not significantly different from that of normal ripe fish (No. 5). (b) The high degree of unsaturation of the C₂₀ and C₂₂ groups is in evidence throughout with no progressive or drastic changes in value. (The corresponding degrees of unsaturation for salmon body fat are about -5 H and -7 H.) (c) As ripening progresses there is a fall in the percentages of C₁₄, C₁₆ and C₂₀ acids, C₂₂ acids remaining practically constant. (d) There is a marked rise in the content of C₁₈ unsaturated acids and a small but significant rise in stearic acid content with development. (e) The degree of unsaturation of the C₁₈ acids steadily decreases from No. 1 to No. 5.

The composition of sample No. 1 is noteworthy in that the percentages of the various acid groups are not significantly different from those of a salmon body fat [Lovern, 1934, 2]. It differs markedly as regards the degrees of unsaturation of the C₁₈, C₂₀ and C₂₂ acids. It was evident during analysis that monoethylenic C₂₀ and C₂₂ acids were absent from all the egg fats. In the body fats they were present in considerable amount. Oleic acid also appeared to be almost absent from sample No. 1. At least two interpretations of these facts are possible: (1) That depot fat is taken, the monoethylenic acids (except palmitoleic acid) selectively removed, and the rest passed on into the ova. (2) That the C₁₈, C₂₀ and C₂₂ monoethylenic acids are desaturated either before the fat is deposited, or else in the ova themselves. Theory (1) is less likely, since it would, in its simplest form, lead to greatly diminished quantities of C₁₈, and especially of C₂₀ and C₂₂, acids, together with a very high content of C₁₄ and C₁₆ acids in the ova. The C₂₂ acids of the ova fat consisted largely of an acid with 6 double bonds. The percentage of such an acid, if any, present in the depot fat, must have been very low. If this acid were selected from the mixed C₂₂ acids available, whereas the C₁₄ and C₁₆ acids were taken in their entirety, the ratio of C₂₂ to C₁₄ or C₁₆ acids in the ova fat would be very much lower than is the case. It is also difficult to picture a mechanism which could remove these three monoethylenic acids so selectively. Whilst not impossible, it is very unlikely that there is any peculiarity in the

¹ "Baggot" is a term applied to ripe female salmon taken early in the year which, for some reason, have not spawned.

glyceride structure of these oils that could lead to certain glycerides being selected containing amongst them all the acids with the exception of monoethylenic C_{18} , C_{20} and C_{22} acids. Theory (2) would account for the observed composition of the egg fats. It has been shown [Lovern, 1935] that fish are probably able to synthesise polyunsaturated acids of 20 and 22 carbon atoms and a desaturation mechanism may possibly be common. It is the rule for fish egg fats to be more unsaturated than the depots from which they are drawn [Lovern, 1934, 2].

Turning to sample No. 2, the greatest change which has occurred since the No. 1 stage is the marked fall in degree of unsaturation of the C_{18} acids. Once again there are two possible explanations: (A) that as development proceeds the desaturation mechanism (or other means of eliminating monoethylenic acids) is confined to the C_{20} and C_{22} acids only, C_{18} going in with about -2.7 H average unsaturation; (B) that actual hydrogenation of the C_{18} acids is taking place. There is no evidence to decide between these theories.

This fall in unsaturation of the C_{18} acids continues throughout the whole series of fats, and there is moreover a steady increase in the content of C_{18} acids. It is obvious, however, that addition of less unsaturated C_{18} acids to a fat of the composition of No. 1 would not in itself account for the observed fall in unsaturation of the total C_{18} group. Considering only Nos. 1 and 2, the added C_{18} to raise the percentage from 23.7 to 25.3 and yet lower the unsaturation from -4 H to -3.2 H would have to have a negative iodine value. Extra C_{18} acids are undoubtedly added to the fat, but in addition either theory (A) or theory (B) above must apply.

The origin of these extra C_{18} acids is obscure. The weight of a roe in sample No. 5 was about 800 g. An average figure for the fat content is 10%. Hence the ova finally contain about 80 g. of fat of composition No. 5. Of this about 8 g. must be "added" C_{18} acids. This must either have come from the depots or have been synthesised from non-fatty sources. It is suggestive that the greatest increase in the C_{18} percentage comes after the fish has been up river for some time. Its state of starvation is already well advanced. In this condition it is unlikely that much non-fatty matter could be spared for fat synthesis, especially when so much depot fat is already available. It was shown previously [Lovern, 1934, 2] that there is no evidence of any selective withdrawal of C_{18} acids from the depots. Such a selection would have to be made by some membrane or organ after the fat had left the depot. It is hard to imagine it taking place from a mixture of glycerides, since simple tri- C_{18} glycerides would not be expected in great amount. If C_{18} acids were picked out from the total acids available in the mobilised depot fat and transferred to the developing ova, then some stearic acid would accompany them. The observed rise in stearic acid from samples Nos. 1 to 5 is in accord with this. Separation of acids according to molecular size has been suggested before [Lovern, 1934, 1].

The addition of extra C_{18} acids to the total should result in a fall in the percentages of all other components. This is the case except for C_{22} acids which remain approximately constant. It has been pointed out previously [Lovern, 1934, 2] that one of the specific salmon peculiarities is a higher C_{22} percentage than usual. That specific properties are deep-rooted is certain. It seems of importance that the salmon egg fat should be a salmon fat, even though having other characteristics. To maintain this almost constant C_{22} acid content in spite of added C_{18} acids, necessarily involves a slightly preferential transfer of C_{22} acids from the depots to the ova.

In the previous work on the mobilisation of salmon depot fat [Lovern, 1934, 2] it was shown that for male salmon, selectivity took the form of a more ready

mobilisation of small molecules. This led to a rise in C_{22} acids in the depots of the starving fish. In the case of the females, the same selectivity appeared but was not so evident, in that C_{22} acids decreased instead of increasing. The reason for this can now be suggested. Male salmon gonads at all stages of development contain less than 1% of fat, so that even if this fat is formed selectively it can have very little effect on the depots. For the females, however, there is now evidence of a preferential withdrawal of C_{22} acids. This must be in spite of the readier mobilisation of small molecules and must be controlled by a different mechanism. Any attempt to calculate the quantity of extra C_{22} acids mobilised and correlate this with the observed changes in the depots would be useless because the depots examined were those of a single fish in each case. Individual peculiarities, together with experimental errors, might lead to wide discrepancies. This theory can only be suggested qualitatively. As in the case of the C_{18} acids, it is difficult to imagine such a mechanism functioning with glycerides, but simpler with acids. In the present case, in contrast to the C_{18} acids, the operation affects the composition of the remaining depot fat and therefore takes place in the depots. There is, moreover, evidence that hydrolysis in the depots is the first stage of mobilisation. This was alluded to in the previous paper [Lovern, 1934, 2] and the marked rise in free acid in the depots of starved fish deserves further investigation. It would be of great interest to determine whether this free acid consisted mainly of the higher acids, as it should do on the theory of hydrolysis followed by preferential removal of the lower acids.

A further point must needs remain unsettled until methods have been perfected for dealing with it. It would be of interest to know whether the added C_{18} acids have been incorporated as a simple triglyceride or have been added to the other acids before converting into mixed glycerides. If the latter, it is further evidence that the fat was mobilised in the form of acids.

Finally, reference must be made to the work of Channon and Saby [1932] on herring ova fat. They found a steady rise in the iodine value of the fat as the ova developed. Whilst iodine values alone are not a reliable guide to what is happening in a fat, this rise would not be expected in view of the salmon results, wherein, apart from the C_{18} peculiarities (which may well be concerned primarily with the fact that salmon eggs are to be laid in fresh-water) there is no reason for the mean iodine value of the whole fat to change appreciably. Experiments have accordingly been made with herring ova in various stages of ripening. Several hundred were taken in each sample. Care was taken to remove contaminating body fat which is always present at certain stages of development when the herring flesh is particularly rich in loosely-held fat. Phosphatides were removed although the amounts were only small. Channon and Saby did not make this separation. The fats were found to have almost the same iodine values throughout, with no evidence of a rise during development.

SUMMARY.

Analyses have been made of the fat of salmon eggs at different stages of maturity. At all stages the degrees of unsaturation of the C_{20} and C_{22} acids are higher than in the depot fat and two possible explanations of this are discussed.

With ripening there is a marked rise in the proportion of C_{18} acids together with a fall in the mean unsaturation of this group. There is as yet insufficient evidence to indicate the mechanism of these changes. The result is that the ova fat finally takes on some of the characteristics of a fresh-water fish fat, whilst still retaining the specific peculiarities of a salmon fat.

There is evidence of a slightly preferential withdrawal of C_{22} acids from the depots and this agrees with observed changes in the depots of starving fish. Male salmon gonads are relatively non-fatty and the different requirements of the sexes during spawning are reflected in the compositions of the depot fats. In contrast to the C_{22} acids, the preferential translocation of C_{18} acids to the ova does not affect the depot fat composition, and the selective process must operate after the fat has been mobilised.

The writer desires to express his thanks to Mr W. J. M. Menzies and Mr P. R. C. Macfarlane of the Fishery Board for Scotland for assistance in the collection of samples and in examining the scales of the fish used, and also to all those who helped by the supply of materials.

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V. SOME CRITICAL REMARKS ON THE DETERMINATION OF ASCORBIC ACID.

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(Received November 18th, 1935.)

TILLMANS, HIRSCH AND HIRSCH [1932] were the first to use 2:6-dichlorophenol-indophenol to determine the antiscorbutic value of vegetables and fruit-juices. Their estimations were carried out in a neutral medium, but this offers difficulties because other substances such as cysteine, glutathione and ferrous salts interfere under these circumstances. Svrbely and Szent-Györgyi [1933], Birch *et al.* [1933] and Wolff *et al.* [1933] carried out the titration in acid medium: the interference of glutathione and ferrous salts is avoided in this way. Cysteine and ergothioneine however reduce the indicator in acid solution. We have indicated a method of removing by the aid of mercuric acetate, cysteine, ergothioneine, glutathione and tannins [Emmerie, 1934, 1; Emmerie and Van Eekelen, 1934].

These substances are precipitated by mercuric acetate whereas the ascorbic acid remains in solution in the reversibly oxidised state. By this method interfering pigments, which sometimes make the estimation impossible, are also removed. We also have found that mercuric acetate precipitates thiosulphate in urine [Van Eekelen, 1934].

To remove the excess of mercuric acetate after the precipitation and to reduce the reversibly oxidised ascorbic acid H_2S is used. Reduction with H_2S is always necessary, because the oxidases which are present in some vegetables are able to change the reduced ascorbic acid into the reversibly oxidised state when the vegetables are ground up [Van Eekelen, 1935, 1].

Before the introduction of the mercuric acetate method it was thoroughly tested on pure ascorbic acid solutions and on blood, urine and vegetables or plant-juices, to which definite quantities of ascorbic acid had been added. The ascorbic acid was always recovered to the extent of at least 94 %. Tauber and Kleiner [1935] and Plaut *et al.* [1935] have also shown that after the mercuric acetate treatment the ascorbic acid was quantitatively recovered.

According to a recent publication of McHenry and Graham [1935] these authors were not able to recover the ascorbic acid quantitatively after mercuric acetate precipitation. They used the method of Tillmans, Hirsch and Jackisch [1932] to remove interfering pigments. The utility of this method is limited and interfering reducing substances are not removed. For this reason it seemed necessary to us once more to direct attention to the precautions which must be taken to obtain satisfactory results with the mercuric acetate method.

1. The solution or extract to which mercuric acetate is added must be slightly acid (p_H about 5). The precipitation of small quantities of cysteine and ergothioneine is more complete in trichloroacetic acid solution than in acetic acid solution; for this reason we always prepare the extract with trichloroacetic acid.

2. Excess of mercuric acetate must be avoided, for this reason the amount of reagent necessary must be determined on a sample.

3. A very important factor is rapid working after addition of the mercuric acetate to avoid irreversible oxidation. It is necessary to centrifuge off the precipitate and the time between adding the mercuric acetate and passing H_2S into the filtrate must not exceed 5–10 min. (see Table III).

4. When working up urine which contains little ascorbic acid 10 ml. urine + 20 ml. mercuric acetate solution (20%) can be taken.

In the following tables we give some results obtained by the mercuric acetate method with pure ascorbic acid solutions and with urine, blood and potatoes to which ascorbic acid has been added.

Table I. *Pure ascorbic acid solutions with different quantities of mercuric acetate. 10 min. after the addition of the mercuric acetate H_2S was passed through to precipitate HgS .*

					Found mg. ascorbic acid	% recovery
10 ml. of ascorbic acid solution					0.48	100
"	"	+ 0.5 ml. 20 % mercuric acetate			0.46	96
"	"	+ 1 ml. " "			0.46	96
"	"	+ 6 ml. " "			0.45	94
"	"	+ 10 ml. " "			0.45	94

Table II. *Addition of pure ascorbic acid to blood, urine and potato and regeneration by H_2S after mercuric acetate precipitation.*

			Found mg. ascorbic acid	% recovery
20 ml. blood			0.13	—
"	+ 1.09 mg. ascorbic acid		1.18	97
10 ml. blood			0.04	—
"	+ 4.92 mg. ascorbic acid		4.91	99
10 ml. urine			0.13	—
"	+ 1.66 mg. ascorbic acid		1.70	95
10 ml. urine			0.06	—
"	+ 2 mg. ascorbic acid		2.02	98
10 gm. potato			3.11	—
"	+ 2.25 mg. ascorbic acid		5.35	100

Table III. *Relation between recovered ascorbic acid and time during which the ascorbic acid has been in contact with mercuric acetate.*

Time in min.					Found mg. ascorbic acid	% recovery
	10 ml. ascorbic acid solution without mercuric acetate				0.43	100
1	"	"	+ 1 ml. 20 % mercuric acetate		0.42	98
5	"	"	" "		0.42	98
10	"	"	" "		0.41	95
20	"	"	" "		0.40	93
30	"	"	" "		0.37	86
60	"	"	" "		0.35	81

Table IV. *Precipitation of thiosulphate with mercuric acetate.*

(9 ml. indicator = 1 mg. ascorbic acid)		ml. indicator
10 ml. urine after mercuric acetate precipitation		1.4
" + thiosulphate without mercuric acetate precipitation		13
" + thiosulphate after mercuric acetate precipitation		1.4

Dewjatnin and Doroschenko [1935] used lead acetate to remove interfering substances.

We have the following objections against this method:

1. The authors do not use H_2S , so that reversibly oxidised ascorbic acid is not estimated.

2. Cysteine is not precipitated by lead acetate.

3. We have found [Emmerie, 1934, 2] that, though pure ascorbic acid solutions are not precipitated by lead acetate in slightly acid or neutral medium, addition of ascorbic acid to urine and precipitation with lead acetate in slightly acid and neutral solution causes great losses of the ascorbic acid.

The reduction of silver nitrate by organs and extracts as a criterion for their ascorbic acid content has proved to be very doubtful, because substances such as cysteine, glutathione and others are able to inhibit the reduction [Huszak, 1933; Emmerie, 1934, 2; Svirbely, 1935]. The quantitative determination of ascorbic acid by the tungstic acid method of Fujita *et al.* [1935] cannot be used in the presence of adrenaline (suprarenal extracts), because adrenaline inhibits the reaction and the values obtained are too low [Van Eekelen, 1935, 2].

SUMMARY.

1. Directions are given for obtaining satisfactory results with the mercuric acetate method of determining the ascorbic acid content of different substances and fluids.

2. Some critical remarks on other methods are made.

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VI. THE TYROSINE METABOLISM OF *BOMBYX MORI* (SILK WORM).

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(Received November 21st, 1935.)

IN view of the high tyrosine content of silk it was of interest to examine quantitatively the tyrosine intake of the silk worm and compare it with that of the silk produced. For this purpose one hundred eggs of the commercial silk worm, *Bombyx mori*, were purchased, from which 85 worms hatched. In six weeks 79 surviving worms had formed cocoons, varying in colour from a pale yellow to an intense orange-yellow. Throughout this period of six weeks the grubs were fed on mulberry leaves obtained from the Chelsea Physic Garden. The leaves were gathered in the morning and placed in a tin with well-fitting lid, weighed and fed to the grubs. The following morning all remains of the previous day's feed were removed, dried for 2 hours in a steam-oven and weighed. The weight of water removed from the mulberry leaves by this procedure was found to be on the average 77.95%, and the weight of leaves consumed by the 79 grubs corresponded to 125.71 g. of "dried leaves". The 79 cocoons and contained grubs were together dried and weighed and a portion was subsequently analysed for tyrosine. Some of the silk alone and also the mulberry leaves were likewise analysed. Both leaves and silk were placed before analysis in a steam-oven for 2 hours. The subsequent weights served as a standard and are referred to as "dried weight" throughout. The dried weight of the 79 cocoons *plus* contained grubs was found to be 21.10 g.

The mortality of 7% was surprisingly low, the dealer having led me to expect one of 50%.

Determination of the tyrosine intake.

Extraction preliminary to hydrolysis. Acetone, methylated spirit and a combination of the two were used in a Soxhlet apparatus to extract about 2 g. of dried leaves. Methylated spirit alone or followed by acetone was found to remove the greatest weight. Extraction of the fresh leaves with boiling methylated spirit in an open vessel proved to be a better method, as much as 32% of the dried weight being extracted as against 21% by the Soxhlet method. Although the chlorophyll and the other leaf pigments appeared to be removed completely, the leaves remained of a greyish tinge.

Hydrolysis of extracted leaves. For the hydrolysis and subsequent estimation of the tyrosine, the method of Folin and Ciocalteu [1927] was followed. The estimations of tyrosine were conducted on the day following the hydrolysis. Considerable difficulty was experienced in matching the unknown with the standard, owing to the yellow colour which persisted even after treatment with kaolin. In two of the following estimations caramel was added to the standard and the colour match was thereby much improved.

The figure given for tyrosine content is in each case the average of at least three colorimetric estimations. The estimations in which caramel was used were thought to be the more accurate. The tyrosine content was taken as the mean of these estimations, giving a value of 1.10 g. tyrosine per 100 g. dried leaves.

Weight of extracted leaves hydrolysed (g.)	Tyrosine content	
	% of dried weight of extracted leaves	% of dried weight of leaves
1.423	1.92	1.49
*2.166	1.59	1.33
*1.315	1.49	1.31
*1.203	1.69	1.23
*†1.206	1.55	1.09
	1.61	1.13
†1.567	1.59	1.07

* Hydrolysate shaken with kaolin. † Caramel added to standard.

Determination of the tyrosine content of silk together with contained grub.

The hydrolysis was performed as for the leaves without preliminary extraction; there was no necessity to employ kaolin or caramel since the colour of the hydrolysate did not interfere with the colour comparison. Some difficulty was experienced owing to the development of turbidities after heating with mercuric sulphate. This was obviated by adopting Folin and Marenzi's [1929] modification of the original Folin and Ciocalteu method [1927].

Hydrolysis number	Dried weight of silk <i>plus</i> grub hydrolysed (g.)	Tyrosine content
		% of dried weight
1	1.044	5.12
2	1.251	6.03
3	1.047	5.23
4	0.862	5.65
5	1.013	5.04
6	1.005	5.75
		Average 5.47
*7	1.043	5.05

* These cocoons were produced by a second batch of grubs whose eggs had been delayed from hatching by being kept in a cold place.

A number of cocoons of *Bombyx mori* from Cyprus were provided by the kindness of Dr Chandler of the Imperial Institute. The tyrosine content of the silk of these cocoons was estimated as above, and also that of the silk of the worms hatched here for purposes of comparison.

	Dried weight hydrolysed (g.)	Tyrosine content % of dried weight
Cyprian silk (1)	0.9915	10.0
(2)	0.9620	10.39
Own silk	0.731	10.3

The tyrosine content of silk of *Bombyx mori* obtained by this method by Silberman and Lewis [1932] was 9.75 % dry weight, or 11 % calculated on an ash-free basis. Whereas analysis of the silk alone gave a value of 10.3 %, analysis of the silk along with the contained grub gave the lower value of 5.47 %. The following figures show the weight of the grubs expressed as a percentage of the total weight of grubs *plus* silk:

Dried weight of six grubs (g.)	Dried weight of six grubs <i>plus</i> their silk (g.)	Dried weight of six grubs: % dried weight of grubs <i>plus</i> silk
2.056	3.497	58.80
2.150	3.645	58.98
1.991	3.310	60.14
1.831	3.028	60.47

Taking the average value of 59.6 g. of grubs to 40.4 g. silk, the grubs contained, by calculation, about 2% tyrosine. The variation in the tyrosine values expressed as a percentage of silk *plus* grubs (*viz.* 5.05 to 6.03%) was expected, since as the above table shows, the grub does not form a constant weight of the cocoon.

*Comparison between tyrosine intake of worms and tyrosine of silk
and contained grub.*

Taking the tyrosine content of silk *plus* grub as 5.47%, the 21.10 g. of silk *plus* grub from the 79 worms contained 1.15 g. tyrosine. The tyrosine content of the leaves was 1.10%, so that the 125.71 g. of leaves ingested by the 79 worms represented a tyrosine intake of 1.38 g. That is considerably more than was found in the silk *plus* grubs. The excess of tyrosine intake over the output for the 79 grubs during the six weeks, was 0.23 g. No estimation was made of the loss of nitrogen by the excreta. In conclusion then, it is probable that the intake of tyrosine in the proteins of the mulberry leaves during the life history of the silk worm is sufficient to account for the high proportion of that amino-acid in the silk produced. There is no need therefore to have recourse to the supposition of tyrosine synthesis on the part of the worms.

SUMMARY.

The tyrosine intake in mulberry leaves ingested by silkworms (*Bombyx mori*) was found to exceed the output of tyrosine in silk and contained grubs. hence there is no need to postulate a mechanism for the synthesis of tyrosine.

I am indebted to Miss M. M. Murray for suggesting this work to me, and for her helpful criticism. This work was carried out by the aid of a grant from the Waller Research Fund.

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VII. THE ISOLATION OF PYRUVIC ACID FROM THE BLOOD OF VITAMIN B₁-DEFICIENT PIGEONS.

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(Received November 26th, 1935.)

THOMPSON AND JOHNSON [1935] have shown that a large increase in the amount of bisulphite-binding substances in the blood is specifically related to vitamin B₁ deficiency in the pigeon. They obtained incomplete evidence that the substance responsible for this increase is pyruvic acid. Enough pyruvic acid for analysis has now been isolated in the form of the 2:4-dinitrophenylhydrazone.

METHOD.

56 g. of blood were collected from eight vitamin B₁-deficient pigeons in head retraction. They had been dosed with glucose once according to the custom of this laboratory. About 1 ml. of 25% trichloroacetic acid was added for every 5 g. blood. The centrifugates were filtered into a large separating funnel, the precipitates were washed once with 5% trichloroacetic acid and the washings filtered into the same funnel. A 0.6% solution of 2:4-dinitrophenylhydrazine was added in the proportion of 5 ml. reagent to 20 ml. of filtrate. After standing a week, the mixture was adjusted to approximately p_{H} 1 with 10*N* NaOH, and the hydrazine and hydrazones were shaken into ethyl acetate. The ethyl acetate phase was shaken with successive portions of Na₂HPO₄ solution, first saturated, then *M*/15, until the aqueous phase was brown coloured, without any yellow tinge. The phases failed to separate sharply, presumably because of the large amounts of fat present. The alkaline solution was adjusted to approximately p_{H} 1 with 10*N* HCl, and the mixture of hydrazones and accompanying impurity of hydrazine were taken up once more in ethyl acetate. The whole procedure of shaking into Na₂HPO₄ solution, acidification and shaking into ethyl acetate was repeated until the last trace of colour could be removed by Na₂HPO₄ solution. The final solution of hydrazone in ethyl acetate was washed five times with equal volumes of *N* HCl and once with distilled water. The ethyl acetate was distilled off *in vacuo* at about 20°, leaving an oily orange residue.

The residue was extracted twice with light petroleum, dried *in vacuo* at room temperature, dissolved in a minimum volume of half-saturated Na₂CO₃ and precipitated by the addition of 10*N* HCl. It was twice more reprecipitated from half-saturated Na₂CO₃ solution. It was then washed twice with very small volumes of *N* HCl and once with distilled water, and dried *in vacuo* over CaCl₂ at room temperature.

The product was recrystallised three times from very small volumes of ethyl acetate and was finally recrystallised twice from ether by the addition of large volumes of light petroleum. It was dried *in vacuo* over CaCl₂ at room temperature.

The yield was 5.5 mg. of a bright yellow compound, m.p. 214° (corrected). (Found: N 21.12%. C₈H₈O₆N₄ requires N 20.88%.)

Another 55 g. of blood from 10 pigeons in head retraction yielded 7.3 mg. of hydrazone m.p. 216° (corrected). (Found: N 20.69%.)

108 g. of blood from 10 normal pigeons yielded 2.9 mg. of a 2:4-dinitrophenylhydrazone melting at 206° (corrected). There was not enough hydrazone for

recrystallisation from ethyl acetate, and most of it was lost in repeated recrystallisation from ether with light petroleum. The m.p. was never higher than 209° (corrected), but in all other properties the substance was similar to the two preparations described above. (Found: N 18.77 %.)

Analysis of 2:4-dinitrophenylhydrazones by the micro-Dumas method is liable to error, the values tending to be low unless the combustion tube is heated longer than for most compounds. The normal blood was worked up before this was realised, and the value is probably about 2.4 % low.

In these experiments 111 g. of blood from 18 vitamin B₁-deficient pigeons gave 12.8 mg. of hydrazone. This is equivalent to 3.8 mg. of pyruvic acid/100 g. of blood. Considering the inevitable losses during purification, this figure agrees satisfactorily with the figures of Table III of Thompson and Johnson [1935], who found an average of 5.65 mg. pyruvic acid/100 g. blood. In the case of normal pigeons, since the 2:4-dinitrophenylhydrazone collected was not pure, it can only be said that not more than 0.9 mg. of pyruvic acid was present in 100 g. blood.

DISCUSSION.

The assumption of Vogt-Møller [1931] that methylglyoxal is the toxic agent in vitamin B₁ deficiency has been verified to some extent by Geiger and Rosenberg [1933], who found methylglyoxal in the urine of avitaminous dogs and rats. This cannot hold for the pigeon. No trace of methylglyoxal-2:4-dinitrophenylbishydrazone was seen in working up the blood of vitamin B₁-deficient pigeons. It would have been detected if it had appeared, since it is one of the most easily identified of all hydrazones.

SUMMARY.

1. Pyruvic acid in the blood of vitamin B₁-deficient pigeons has been identified by preparation and analysis of the 2:4-dinitrophenylhydrazone.
2. In the case of normal pigeons, pyruvic acid has not been identified with certainty.

I am deeply grateful to Prof. R. A. Peters for the interest he has shown in this work.

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VIII. α -GLYCEROPHOSPHORIC ACID AND BRAIN METABOLISM.

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ASHFORD AND HOLMES [1931] found that sodium glycerophosphate increases the oxygen uptake of chopped brain tissue from rabbits, and Davies and Quastel [1932] that it can donate hydrogen to methylene blue in the presence of bullock's brain. In neither of these researches was the isomeride specified. Peters and Sinclair [1933, 1] showed that α -, but not β -, glycerophosphate increases the oxygen uptake of normal pigeon's brain, and [1933, 2] that the effect of pyruvic acid on the extra respiration of the vitamin B₁-deficient brain due to α -glycerophosphate is apparently inconsistent with the scheme of Embden and Meyerhof. The importance of this scheme as a mechanism for glycolysis justified a study of the metabolism of α -glycerophosphate in the brain.

The present work is in two sections, the first dealing with factors that influence the removal of sodium α -glycerophosphate by the brain, the second with the oxidation products of α -glycerophosphate.

THE UTILISATION OF α -GLYCEROPHOSPHATE BY BRAIN TISSUE *IN VITRO*.

The following description applies to all the respiration experiments described.

Normal pigeons, kept on the customary diet of this laboratory, were used throughout. Each bird was guillotined, its cerebrum transferred to a porcelain plate and minced with a bone spatula. Samples of about 150 mg. were placed in duplicate weighed Barcroft bottles of the standard type containing 3.0 ml. of fluid, including Ringer-phosphate solution and additions of substrate. O₂ uptakes at $38 \pm 0.1^\circ$ were then determined in the usual way. Shaking was carried out at 60-80 complete revolutions per min., since higher speeds gave bad duplicates by washing tissue on to the sides of the bottle.

At the end of the period of respiration the bottles were taken from the bath and grease was wiped from the necks with a clean duster. When the bottles were cool, the appropriate protein precipitant was added.

The various substrates have been prepared and used as follows.

Lactate. Pure zinc *l*-lactate was decomposed with NaHCO₃. The final concentration in the Ringer-phosphate was 0.033 *M*.

Pyruvate. A commercial preparation was repeatedly distilled *in vacuo* until the fraction at 50° was colourless. It was made up to 10 *M* with water and kept at 4°. It was mixed with the Ringer-phosphate in a concentration of 0.019 *M*.

α -Glycerophosphate. Sodium α -glycerophosphate was obtained in solution by treating a sample of Boots's calcium α -glycerophosphate [King and Pyman, 1914] with the theoretical amount of sodium oxalate. The solution was alternately cooled to 0° and heated to 100° until decomposition was complete. The centrifugate always gave negative tests for Ca and oxalate.

Pyrophosphate. A sample of A.R. Na₄P₂O₇ was twice recrystallised from water. Just before use it was brought to red heat for 10 min. The required amount was dissolved in a little boiling water, the *p*_H was adjusted to 7.3 with 10*N* HCl, and the Na₄P₂O₇ was added to the Ringer-phosphate in a final concentration of 0.013 *M*.

Ringer-phosphate solution. NaCl, 0.13 *M*; KCl, 0.0025 *M*; NaHCO₃, 0.0014 *M*; KH₂PO₄, 0.1 *M*; enough NaOH (about 0.068 *M*) to bring the *p*_H to 7.3. Glass-distilled water was used.

After all the additions of substrates had been made, the *p*_H was adjusted to 7.3 by the addition of NaOH or HCl as needed.

1. Rate of disappearance of α -glycerophosphate.

The effects of lactate and pyrophosphate upon the utilisation of α -glycerophosphate were first studied, since lactate is probably a normal metabolite in the pigeon's brain and sodium pyrophosphate profoundly modifies the brain's respiration *in vitro* [Peters and Sinclair, 1933, 2]. The results of eleven pairs of experiments are typified by the two experiments given below.

Oxygen uptake of brain in presence of various substrates.

(Expressed as μ l. O₂/g. tissue/hr. in the period indicated. Average of duplicates.)

Additions	0-1 hr.	1-2 hr.	2-3 hr.	3-4 hr.	4-5 hr.	5-6 hr.
Exp. 54. Pyrophosphate not added						
None	910	705	510	600	395	300
α -GP	1430	1095	905	925	615	555
L	2045	1830	1085	990	685	560
L + α -GP	2430	1960	1515	1630	1360	1240
(α -GP) - (Residual)	+ 520	+ 390	+ 395	+ 325	+ 220	+ 255
(L + α -GP) - (L)	- 215	+ 130	+ 430	+ 640	+ 675	+ 680
Exp. 72. Pyrophosphate added						
None	1245	1025	670	475	425	250
α -GP	1775	1395	1110	810	660	550
L	2950	2640	2320	1995	1830	1660
L + α -GP	3495	3035	2635	2360	2075	2050
(α -GP) - (Residual)	+ 530	+ 370	+ 440	+ 335	+ 235	+ 300
(L + α -GP) - (L)	+ 545	+ 395	+ 315	+ 365	+ 245	+ 390

When pyrophosphate has been added, the extra oxygen uptake due to α -glycerophosphate is additive to that due to lactate. The four curves are parallel. When pyrophosphate has not been added, the curve for lactate alone starts at a high level and falls off rapidly, but the curve for lactate *plus* α -glycerophosphate starts high and is maintained throughout. There seems to be an interaction between lactate and α -glycerophosphate. An alternative to actual interaction might be that the brain forms pyrophosphate from α -glycerophosphate, and that this pyrophosphate then maintains the extra respiration due to lactate. The effect can be seen in the data of Peters and Sinclair [1933, 1], who however did not remark upon it.

Estimations of the rate of disappearance of α -glycerophosphate were made by a method similar to that described by Meyerhof and Kiessling [1933].

The proteins were precipitated with trichloroacetic acid. Inorganic phosphates were precipitated from the filtrate with baryta and barium chloride at *p*_H 10. Five volumes of 97% ethyl alcohol were added to the centrifugate and the crude precipitate of Ba α -glycerophosphate was dried *in vacuo*. Aliquot parts were taken for analysis according to Pregl [1930, pp. 180-90].

The sample was weighed into tin cups and treated with HI. The isopropyl iodide thus formed was decomposed in alcoholic silver nitrate. The silver iodide was filtered off, washed, dried and weighed in the halogen filter stick.

The method was checked against vanillin and pure Ca α -glycerophosphate. Occasional analyses of vanillin were made to insure against faulty reagents.

The maximum error in recovery of known small amounts of Ca α -glycerophosphate added to brain tissue was 5%. In calculating the results no correction

has been made for variations of tissue weight in different bottles since the tissue blank is negligible. There is something estimated as α -glycerophosphate in the brain immediately after death, but in five cases it did not amount to more than 0.023 mg. of Ca α -glycerophosphate per g. of tissue. Single estimations only were made on the precipitates of the impure Ba α -glycerophosphate, because the error of the final analysis was not more than 1% and the error in recovery could amount to 5%. The increased accuracy obtained by duplicate estimations was therefore not commensurate with the extra time spent.

The results are collected in Table I.

Table I. *Rate of disappearance of α -glycerophosphate.*

(Calculated as mg. Ca α -glycerophosphate/g. tissue/hr.)

With added pyrophosphate			Without added pyrophosphate		
Exp.	α -GP alone	α -GP + lactate	Exp.	α -GP alone	α -GP + lactate
71	17.7	12.8	62	6.3	5.6
72	11.2	9.6	63	3.3	3.6
73	22.2	—	64	2.1	2.1
79	12.1	14.1	65	3.4	8.8
81	19.1	18.2	66	3.9	7.0
84	20.4	25.2	67	3.4	3.2
85	—	14.0	70	4.3	7.0
90	13.4	—	87	5.4	3.1
91	13.9	—	88	7.0	—
Average	16.3	15.7		4.3	5.1

Added $\text{Na}_4\text{P}_2\text{O}_7$ causes a large increase in the rate of α -glycerophosphate disappearance, but the addition of lactate has little effect. In complete combustion 1 mol. of O_2 would remove 0.15 mol. of α -glycerophosphate. Table II is a calculation of the ratio mols. α -glycerophosphate removed/mols. extra O_2 uptake due to added α -glycerophosphate. It shows that only a small part of the α -glycerophosphate could be oxidised completely and, since the ratio varies from 1.1 to 8.8, that the oxidation involved is not a simple one. $\text{Na}_4\text{P}_2\text{O}_7$ doubles the ratio. The effect of pyrophosphate may be connected with the activity of adenylyl pyrophosphate as coenzyme to yeast glycerophosphate dehydrogenase [Lehmann, 1934].

2. *The independence of pyruvate and α -glycerophosphate.*

If the Embden-Meyerhof scheme were valid for pigeon's brain tissue, the rate of disappearance of α -glycerophosphate *in vitro* should be increased by added pyruvate. This was tested under anaerobic conditions, with and without added pyrophosphate.

Additions to the Barcroft bottles were made as follows: α -glycerophosphate alone, pyruvate alone and α -glycerophosphate *plus* pyruvate. The α -glycerophosphate in the trichloroacetic extracts was estimated as before and the pyruvate by the bisulphite-iodine titration of Clift and Cook [1932]. For anaerobic experiments, nitrogen was used instead of oxygen. The experiments all ran for 2½ hours.

Pyruvic acid used in this way does not interfere with the estimation of α -glycerophosphate, but complications arose because aerobically, but not anaerobically, α -glycerophosphate gives rise to a small amount of bisulphite-binding substance, sometimes equivalent to as much as 0.5 mg. pyruvic acid/g. of tissue in 2½ hours. The assumption was made that the amount of this substance is not affected by the presence of pyruvate, and it was added as a blank. No correction was made for variations in tissue weight.

Table II shows that there is no interaction between pyruvate and α -glycerophosphate, aerobically or anaerobically, with or without added pyrophosphate.

Table II. *Effect of pyruvate on removal of α -glycerophosphate.*

Exp.	Substrates added	α -Glycerophosphate removed As mg. Ca salt/g. tissue/hr.			Pyruvate removed As mg. acid/g. tissue/hr.		
		α -GP	α -GP + pyr.	Diff.	Pyr.	α -GP + pyr.	Diff.
		Aerobically					
87	Pyrophosphate not added	5.4	5.95	+ 0.55	5.27	5.08	- 0.19
88	" "	7.0	7.7	+ 0.7	2.35	2.08	- 0.27
89	" "	3.0	3.6	+ 0.6	5.52	4.84	- 0.68
90	Pyrophosphate added	13.45	11.6	- 1.85	4.16	4.33	+ 0.17
91	" "	13.9	13.2	- 0.7	4.82	3.10	- 1.72
Anaerobically							
93	Pyrophosphate not added	0.2	0.1	- 0.1	1.65	0.79	- 0.86
94	" "	1.9	1.3	- 0.6	5.02	4.58	- 0.44
96	Pyrophosphate added	2.35	1.6	- 0.75	1.83	1.88	+ 0.05
97	" "	1.75	1.0	- 0.75	2.48	2.13	- 0.35

To test the assumption that bisulphite-binding substances are produced aerobically from α -glycerophosphate in equal amounts with or without added pyruvate, estimation of the pyruvate was made in eight experiments by the Neuberg-Case 2:4-dinitrophenylhydrazine method, as modified by Peters and Thompson [1934]. The results (Table III) agree with those in Table II. In this method the bisulphite-binding substance formed from α -glycerophosphate does not appear as a blank.

Table III. *Disappearance of pyruvic acid as estimated by extraction of the 2:4-dinitrophenylhydrazone.*

(As mg. acid/g. tissue/hr.)

	Substrates	Pyruvate alone	Pyruvate + α -glycerophosphate	Difference
Aerobically				
1	Pyrophosphate not added	5.08	1.72	- 3.36
2	" "	3.82	3.56	- 0.26
3	" "	7.19	2.16	- 5.03
4	Pyrophosphate added	5.63	6.28	+ 0.65
5	" "	6.11	5.94	- 0.17
6	" "	3.90	4.36	+ 0.47
Anaerobically				
7	Pyrophosphate not added	3.25	3.08	- 0.17
8	Pyrophosphate added	3.08	1.95	- 1.13

It can be seen from these figures that the disappearance of α -glycerophosphate is largely dependent upon the presence of oxygen.

Only two anaerobic experiments were made by the second method, which is much more specific than the first, but the complete agreement between the two sets of experiments strengthens the conclusion that the Embden-Meyerhof scheme does not hold for pigeon's brain tissue with or without added pyrophosphate, aerobically or anaerobically. If anything, the two compounds exercise a sparing action on each other.

The α -glycerophosphate and pyruvate were added in concentrations which had submaximum effects on the respiration. Any interaction would have

appeared in the analyses. The Ringer-phosphate solution was $N/10$ with respect to potassium, a concentration at which the effect on brain glycolysis [Ashford and Dixon, 1935] is maximum. However, as no experiments were made with potassium-free Ringer solution, the effect of potassium on the results is unknown.

No attempt has been made to show that α -glycerophosphoric acid is a normal metabolite in the pigeon's brain, although a very small amount of something estimated as α -glycerophosphate is present in the tissue immediately after death. The work does indicate that the Embden-Meyerhof scheme cannot hold for the normal pigeon's brain. The dual mechanism for brain glycolysis suggested by Ashford and Holmes [1929] and Ashford [1933] implies that the Embden-Meyerhof scheme could hold only for a part of the lactic acid production. Ashford [1933], in two experiments with slices of rabbit's brain, found that the lactic acid produced from the combination of pyruvate and α -glycerophosphate was less than that from pyruvate alone.

Peters and Thompson [1934] favoured the view that the disappearance of pyruvic acid in the pigeon's brain is dependent upon some other change. Peters *et al.* [1935] found that the actions of fluoride and iodoacetate on vitamin B_1 -deficient pigeon's brain tissue are consistent with the Embden-Meyerhof scheme so far as pyruvic acid is concerned. In the present experiments α -glycerophosphoric acid did not behave in brain as it does in muscle. In experiments with avitaminous brain by Johnson and Peters [unpublished], vitamin B_1 had no influence upon the disappearance of α -glycerophosphoric acid, though it is known to affect pyruvate. The evidence therefore suggests that in the pigeon's brain there is a glycolytic cycle, but that α -glycerophosphoric acid is not a part of it.

THE OXIDATION PRODUCTS OF α -GLYCEROPHOSPHATE.

Brain tissue, in contrast to muscle, seems to utilise α -glycerophosphate in appreciable quantity only in the presence of oxygen. It has been suggested that glycerophosphate dehydrogenase produces glyceraldehyde-3-phosphoric acid [Harrison, 1935; Fischer and Baier, 1932], but it is possible that other carbonyl compounds are formed as well. It seemed practicable to detect them by their reducing properties, and a study was accordingly made of the filtrates from brain tissue which had respired in the presence of α -glycerophosphate.

Trichloroacetic acid was unsuitable as a protein precipitant because, when boiled in alkaline solution, it decomposed and formed reducing substances. Samples of Merck, Kahlbaum and B.D.H. trichloroacetic acid all did this. After much experimentation with different substances, zinc, which has frequently been used for this purpose [see especially Somogyi, 1929; 1930], was found to be suitable. In these estimations $0.4\ M\ ZnSO_4 \cdot 7H_2O$ in $2N\ H_2SO_4$ was used. It had many advantages: precipitation was carried out in the cold; the blank remained very small even when filtrates were boiled for 5 hours; and glycogen, hexosediphosphate, Robison's hexosemonophosphate and all the common mono- and di-saccharides were soluble in it.

The respiration period was usually 2 hours. The contents of duplicate Barcroft bottles were poured into centrifuge-tubes containing 2 ml. of the zinc solution and the bottles were washed out three times with 2 ml. portions of distilled water. After standing half an hour with occasional mixing, the precipitates were centrifuged and the centrifugates were filtered into 100 ml. volumetric flasks through Whatman 44 papers previously washed three times with boiling distilled water. (It was found that even the best papers contain water-soluble reducing substances in appreciable amounts.) The precipitates were ground with 0.5 ml. of the zinc solution, the centrifuge-tubes were washed down with 3 ml. of water, the centrifugates filtered into the appropriate 100 ml. flask, and the process was repeated once. The filter-papers were washed once with 5 ml.

of distilled water. When the solutions had been made up to 100 ml., duplicate samples of 10 or 20 ml. were pipetted into boiling-tubes fitted with Kjeldahl bulbs to prevent reoxidation. From this point estimations of the reducing substance were made by the method of Hagedorn and Jensen [1923].

In estimating pure glucose, it was found that reduction of the ferricyanide was complete only at p_H 10 or above. The filtrates were therefore adjusted to p_H 11 with NaOH just before reduction. Recovery of glucose was consistently 97 %.

Table IV shows that added lactate is not converted into reducing substances by normal brain tissue under these conditions:

Table IV. *Reducing substances produced by normal brain.*

(Expressed as mg. glucose/g. tissue.)			
Exp.	Lactate not added	Lactate added	Difference
Pyrophosphate not added			
7	2.3	2.35	+ 0.05
15	1.7	1.7	0.0
30	7.4	7.35	- 0.05
33	8.45	8.4	- 0.05
34	10.9	10.85	- 0.05
35	6.75	7.1	+ 0.35
36	8.3	7.85	- 0.45
37	10.65	11.4	+ 0.75
40	3.7	3.75	+ 0.05
43	4.2	4.3	+ 0.1
Average	6.40	6.50	+ 0.05
Pyrophosphate added			
41	4.1	4.0	- 0.1
44	4.5	4.5	0.0
46	4.2	4.05	- 0.15
47	5.45	5.7	+ 0.25
Average	4.55	4.55	0.00

Added $Na_4P_2O_7$ does not cause the formation of reducing substance from lactate. No attempt has been made to identify the reducing substance actually estimated, although 10 % of the value is due to substance which reduces at 20° .

To make sure that non-reducing carbohydrates were absent, hydrolysis curves were constructed for the two types of filtrate. The filtrates were made up to 100 ml. at a concentration of $2N H_2SO_4$. Samples were pipetted into boiling-tubes and heated at 100° for varying lengths of time. No great increase in reducing power was seen in five experiments, of which the following is one:

Reducing substances present after hydrolysis.

(Expressed as mg. glucose/g. tissue.)								
Time in hours	...	0	$\frac{1}{4}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3
No addition		2.0	2.05	2.3	2.6	2.85	2.8	2.8
Lactate added		2.2	2.3	2.35	2.6	2.8	2.8	2.85

Reducing substances or potential reducing substances are therefore not formed oxidatively from lactate by normal pigeon's brain tissue *in vitro*. This is in agreement with work by Ashford and Holmes [1931], who found no evidence of synthesis of glycogen or free carbohydrate by chopped rabbit's brain tissue in the presence of lactate.

It was then possible to discover if α -glycerophosphate is changed oxidatively into reducing substances by normal brain tissue. Exactly the same technique

was used as before, since α -glycerophosphate itself has no reducing properties, and lactate is not changed into reducing substances. The results are given in Table V.

Table V. *Production of reducing substances from α -glycerophosphate.*

(Expressed as mg. glucose/g. tissue.)

Pyrophosphate not added				Pyrophosphate added			
Exp.	Lactate	Lactate + α -GP	Difference	Exp.	Lactate	Lactate + α -GP	Difference
54	3.5	4.3	+ 0.8	44	4.5	5.1	+ 0.6
55	4.9	6.9	+ 2.0	45	3.4	4.2	+ 0.8
56	4.2	5.3	+ 1.1	46	4.0	6.2	+ 2.2
57	4.0	5.0	+ 1.0	47	5.7	6.9	+ 1.2
58	6.8	14.6	+ 7.8	49	3.6	5.3	+ 1.7
				50	4.6	5.6	+ 1.0
Average	4.7	7.2	+ 2.5		4.3	5.6	+ 1.3

Fairly large amounts of reducing substance are formed from the added α -glycerophosphate, and pyrophosphate has little effect on this formation. Hydrolysis did not increase the reducing power of the filtrates, and the substance cannot be a polysaccharide or other substance which increases in reducing power on hydrolysis.

Peters [personal communication] noticed that an orange-red precipitate formed slowly when trichloroacetic acid filtrates of brain tissue which had respired with α -glycerophosphate stood for 1 or 2 days with 2:4-dinitrophenylhydrazine. This observation has been confirmed and the precipitate has been partially fractionated.

Aerobic and anaerobic experiments were made with and without added lactate, pyrophosphate and α -glycerophosphate. The red precipitate appeared in large quantities only when α -glycerophosphate had been used and the experiment was aerobic. Lactate and pyrophosphate seemed to have no effect on its appearance. It was therefore formed from an oxidation product of α -glycerophosphate. In order to obtain enough to analyse, large scale experiments were made.

The brains of three normal pigeons were removed, minced and shaken for 3 hours in an atmosphere of oxygen at 38° with 100 ml. of Ringer-phosphate containing α -glycerophosphate in the usual concentration. 20 ml. of 25% trichloroacetic acid were added and the precipitate was centrifuged. 25 ml. of 0.6% 2:4-dinitrophenylhydrazine in 2N HCl were added to the centrifugate and the mixture was kept at 38° for 36 hours. To remove unchanged hydrazine, the orange-red precipitate was centrifuged down and washed with boiling N HCl until the washings were colourless. The precipitate was transferred to a 25 ml. centrifuge tube and was washed with 3 ml. portions of boiling 75% alcohol N with respect to HCl until only a faint orange colour came out in the washings, 250 ml. of which, containing one fraction of the precipitate, were collected.

The residue was dissolved in pyridine and reprecipitated by the addition of 10 volumes of absolute alcohol. This was repeated three times. The product was washed five times with cold absolute alcohol and was dried *in vacuo* over CaCl_2 . The yield was 3.1 mg.

The melting-point was 299° (corr.), the melting-point of methylglyoxal-2:4-dinitrophenylbishydrazone [Barrenscheen and Dreguss, 1931]. It gave a violet colour with alcoholic potash. (Found: N, 26.01%; calc. N, 25.92%.)

Another preparation melted at 298° (corr.) and had N, 25.78%.

The product was therefore methylglyoxal-2:4-dinitrophenylbishydrazone.

The washings collected in the first fractionation were adjusted to p_H 7 with 10N NaOH and were evaporated to dryness *in vacuo* at 40°. To remove inorganic salts and unchanged hydrazine, the residue was washed with boiling N HCl until the washings were colourless. The precipitate was dissolved in boiling absolute alcohol and an insoluble residue was centrifuged. Five volumes of cold N HCl were added to the centrifugate and the precipitate was recrystallised four times from hot absolute alcohol by the addition of cold N HCl. Its melting-point remained constant at 267° (corr.) after recrystallisation from absolute alcohol. It was dried at 120° *in vacuo*.

The crystals had a striking ruby colour. They were insoluble in water but dissolved readily in ethyl acetate and less readily in absolute alcohol. They contained no P or Cl. They gave a violet colour with alcoholic potash and were not acidic in character. (Found: C, 46.27; H, 7.01; N, 15.01 %.)

Another preparation was made in a different way. Cold N HCl was added to the washings from the first fractionation as above. The precipitate was extracted with boiling 75% alcohol N with respect to HCl until only a faint orange colour came out in the washings. The mixture was precipitated again by cold N HCl, and the process was repeated until boiling 75% alcohol N in HCl dissolved all the precipitate. The final precipitate was then extracted with boiling 30% alcohol N with respect to HCl, and the residue was recrystallised from absolute alcohol by the addition of cold N HCl, as above. The properties of this substance were the same as before, but the melting-point remained constant at 262° after recrystallisation. (Found for two preparations by this method: C, 45.58, 43.93; H, 5.93, 6.03; N, 15.98, 16.90 %.)

0.143 mg. substance was mixed with 3.523 mg. camphor. Δ -5.0°; mol. wt. 308.

It has been very difficult to prepare enough of the ruby-coloured compound for analysis, and the melting-points show that at least two of the samples were impure. The analyses agree well enough to allow a calculation of the empirical formula. The assumption has been made that the compound is a 2:4-dinitrophenylhydrazone and contains four nitrogen atoms.

Sample	Mol. wt.	% N	% C	% H	% O	M.P. (corrected)
I	—	15.01	46.27	7.93	30.81	267°
II	—	15.98	45.58	5.93	32.51	262°
III	308 ± 30	16.90	43.93	6.03	33.14	269°

Required for:

$C_6H_{16}O_3 \cdot C_6H_4O_4N_4$	316	17.72	45.57	6.33	30.38	—
$C_6H_{16}O_3 \cdot C_6H_4O_4N_4$	332	16.87	43.37	6.02	33.73	—
$C_7H_{16}O_3 \cdot C_6H_4O_4N_4$	344	16.28	45.35	5.81	32.56	—

Preparation III was probably the purest, and the analysis agrees well with the formula $C_6H_{16}O_3 \cdot C_6H_4O_4N_4$. There is little evidence to show its structure. It gives a violet colour with alcoholic KOH like many monose 2:4-dinitrophenylhydrazones, is not acidic since it is insoluble in half-saturated Na_2CO_3 and is completely insoluble in water. The melting-points of 2:4-dinitrophenylhydrazones follow no general rule, so that conclusions cannot be drawn from its very high melting-point. The most likely compound to form such a hydrazone would be a carbonyl compound of high molecular weight.

The compound appears only when the tissue has been incubated aerobically with α -glycerophosphate, but there is nothing yet to show whether it is formed directly from α -glycerophosphate or is produced from oxidation products of α -glycerophosphate by the action of the reagents used in its isolation. The methylglyoxal is certainly formed in this fashion, since there is only a slight turbidity in the mixture of trichloroacetic acid extract with 2:4-dinitrophenylhydrazine, even after 2 hours at 38°. Furthermore, after 12 hours, if the precipitate is centrifuged, methylglyoxal-2:4-dinitrophenylbishydrazone will continue to precipitate for another 12 hours. The compound forming the ruby-coloured hydrazone may very well be a condensation product of some precursor,

and analogous to the α -keto- γ -valerolactone- γ -carboxylic acid into which pyruvic acid polymerises [Wolff, 1899].

The ruby-coloured compound and methylglyoxal osazone account for only a small part of the α -glycerophosphate which actually disappears. The maximum yields, 4.0 mg. and 12.1 mg., respectively, were obtained in an experiment using 300 mg. of α -glycerophosphate and 3.7 g. of brain tissue, the incubation period being 3 hours. Since calculations show that 150 mg. of the α -glycerophosphate would have disappeared during the time, only 3% of the α -glycerophosphate could be accounted for in this way unless the compounds actually isolated are indicative of true intermediates in the oxidation of α -glycerophosphate.

Two other fractions, which have not been identified, were found in small quantities in the working up of the 2:4-dinitrophenylhydrazones. One is soluble in water, is yellow, gives a red colour with alcoholic potash and melts at 128° (corr.). It is like glyceraldehyde-2:4-dinitrophenylhydrazone. The other accompanies the ruby-coloured compound. It is insoluble in water, soluble in 30% alcohol *N* with respect to HCl, melts at 178° (corr.), and gives a violet colour with alcoholic KOH.

There is as yet no proof that the brain can oxidatively synthesise long carbon chains from short, although a high Meyerhof quotient has frequently been obtained with brain tissue [Warburg *et al.*, 1924]. Ashford and Holmes [1929] and Holmes and Ashford [1930] could show no synthesis of glycogen or other carbohydrates from lactic acid. The present work confirms this for the pigeon's brain so far as lactic acid is concerned. If, however, the 6-carbon atom compound described above were really synthesised by the brain from α -glycerophosphate, and even if α -glycerophosphate were an abnormal metabolite, such a formation would demonstrate oxidative synthesis under special circumstances.

SUMMARY.

The metabolism of α -glycerophosphoric acid in the brain of normal pigeons has been investigated *in vitro* by means of a mince incubated with various substrates in Ringer solution buffered with potassium phosphate.

1. The rate of aerobic removal of sodium α -glycerophosphate is unaffected by added sodium lactate, but is increased by added sodium pyrophosphate.
2. Added sodium pyruvate has no effect upon the aerobic or anaerobic removal of α -glycerophosphate, with or without added pyrophosphate. Therefore the Embden-Meyerhof scheme does not hold for the pigeon's brain.
3. There is aerobic production of reducing substances from α -glycerophosphate.
4. From solutions in which brain tissue has respired with α -glycerophosphate the 2:4-dinitrophenylbishydrazone of methylglyoxal and the 2:4-dinitrophenylhydrazone of a compound of probably six carbon atoms can be prepared.
5. The possibility is discussed that the brain may synthesise oxidatively a compound of six carbon atoms from α -glycerophosphate.

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IX. MAINTENANCE NUTRITION IN THE PIGEON. VITAMIN B₃ CONCENTRATES.

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VITAMIN B₃ may be defined as a factor required for full weight restoration in the pigeon on a diet which, in addition to vitamins B₁ and B₅, includes an adequate supply of basal dietary constituents such as salts and protein. We have previously shown [Carter and O'Brien, 1935] that one of the factors involved in the failure of pigeons on a rice diet to recover normal weight is a partial deficiency of protein. Further, the weight recovery of birds on a diet adequate as regards vitamins B₁ and B₅ and protein is influenced by the severity of the depletion to which they are subjected on rice. It was observed that, whereas birds subjected to moderate depletion of stored vitamins recovered weight completely on receiving supplements of vitamins B₁ and B₅ and protein, with more drastic depletion many birds failed to regain their maximum weight on this diet. This may explain the differences between the results of Morris [1933] and those of Waterman and Ammerman [1935]. The former was led to question the existence of vitamin B₃ on the ground that normal weight could be attained on a diet of autoclaved wheat and 6-16 times the curative dose of vitamin B₁. The latter workers found that, on the same basal diet, pigeons receiving up to 160 γ of crystalline vitamin B₁ daily attained only 80-90 % of their normal weight and concluded that a further factor was required for full weight recovery. In those birds which fail to regain their maximum weight on a diet with adequate protein we have found that full weight restoration was made on administration of an extract of liver which we consider to supply vitamin B₃.

The present paper gives further evidence of the character of this supplement from liver.

EXPERIMENTAL.

Preparation of vitamin B₃ concentrates.

The liver concentrate was prepared as follows: 2 sheep livers weighing about 2 kg. were freed as far as possible from adherent fat and connective tissue. After mincing, the liver was suspended in 6 l. of 97 % alcohol and kept with occasional stirring for 48 hrs. The alcoholic extract was filtered off and the residue again extracted with an equal volume of alcohol. The combined filtrates containing most of the fat were concentrated *in vacuo* at 60° to a small volume. 200 ml. ether were then added, the whole readily dissolving to give a reddish solution. This was shaken in a separating funnel with 200 ml. of water, the aqueous phase removed and the extraction of the ether repeated twice. During this process a bulky white precipitate appeared in the aqueous phase. The aqueous extracts were combined and reserved. The liver residue was again twice extracted with 4 l. 50 % alcohol and the combined filtrates which possessed a strong yellow-green fluorescence were concentrated *in vacuo* at 60° to about 200 ml. This concentrate was shaken two or three times with ether and added

to the solution reserved from the extraction with 97% alcohol. The turbid solution was treated with an equal volume of acetone and kept overnight. After filtration it was concentrated *in vacuo* to a volume such that 1 ml. was equivalent to 20 g. liver (fraction A).

The marked yellow-green fluorescence of fraction A suggested the presence of hepatoflavin and further fractionation was made following the procedure for the isolation of flavins [Kuhn *et al.*, 1933]. 250 ml. of fraction A were treated with 12.5 ml. conc. HCl and 15 g. of fuller's earth added. The mixture was kept for 12 hours with repeated stirring and was then filtered on a Büchner funnel. The earth was extracted 3 times with 200 ml. of a mixture of pyridine, methyl alcohol and water in the ratio 1 : 1 : 4. The pyridine extracts were concentrated *in vacuo* to a syrup which was taken up in 20 ml. of water. On addition of an equal volume of methyl alcohol and 1–2 drops of acetic acid precipitation of fuller's earth occurred. After filtration the solution was concentrated *in vacuo* nearly to dryness and the residue dissolved in hot water. The solution containing flavin was further purified by adsorption upon lead sulphide [Ellinger and Koschura, 1933]. The sulphide was repeatedly extracted with hot water and the eluate concentrated to a volume such that 1 ml. was equivalent to 20 g. liver (fraction F). This fraction possessed a marked flavin fluorescence with only traces of a blue-fluorescent substance. The filtrate obtained after the treatment with fuller's earth was neutralised to p_{H} 4.0 with 20% NaOH and concentrated *in vacuo* so that 1 ml. was equivalent to 20 g. liver (fraction B). This preparation showed in the ultraviolet a strong blue fluorescence which persisted in acid and alkaline solutions. The operations described above were conducted with minimum exposure to light.

Results.

The procedure followed was that of Carter and O'Brien [1935], *i.e.* birds were maintained on a rice diet supplemented with 12 doses of a vitamin B₁ concentrate and 2 g. of caseinogen daily. Approximately 60% of the birds failed to regain their maximum weight on this diet, the remainder recovering their initial weight completely. This unfortunately limits the number of birds available as suitable material for testing the potency of vitamin B₃ preparations. The group of birds selected for these tests had maintained for periods of 11–220 days a stationary weight level which varied within the limits of +5 to –16 g. before administration of liver concentrates. Following administration of the liver concentrates an initial daily rise of 1.0–4.0 g. was recorded, although later as maximum weight was approached the rise became more gradual. This comparatively small rise in weight as a result of the administration of vitamin B₃ may be due to the fact that a level of 80–90% of the maximum had already been attained on vitamin B₁ and caseinogen. This may be attributed either to considerable powers of storage of vitamin B₃ in the bird or to the possibility, not yet excluded, that the vitamin B₁ concentrate carries some of the activity present in the liver preparation. The significance of the results lies, in our opinion, in the restoration of maximum weight in the majority of cases.

Three liver preparations were tested in daily doses equivalent to 10–30 g. liver: fractions A, B and F, and fractions B and F combined. In Table I, the response of 6 birds to fraction F is shown. In two cases, birds 247 and 288, there was a rise nearly to maximum weight and no further improvement occurred when they received whole wheat. In three cases the response was negligible. No further increase in weight was observed on doubling the dose of fraction F. From Table I it is also seen that the effect of fraction B is insignificant. A more

Table I. *Effect of liver supplements.*

Bird	Max. wt. g.	Depletion wt. ex- pressed as % of max. wt.	Stationary wt. period on vitamin B ₁ and caseinogen (days)	Wts. before and after administra- tion of liver suppl. (g.)		Duration of suppl. (days)	Gain or loss in wt. g.	Av. daily gain in wt. g.
Fraction F								
247*	428	73	54	390	412	5	22	4.4
288	402	72	222	363	395	19	32	1.7
299*	398	70	120	369	379	15	10	0.6
292	477	71	49	437	440	9	3	0.3
293*	400	56	11	366	362	11	-4	Nil
822*	426	64	100	406	401	9	-5	Nil
Fraction B								
91*	356	56	115	316	323	10	7	0.7
287*	474	72	113	433	434	9	1	Nil
247*	428	73	119	392	389	10	-3	Nil
190*	500	75	41	440	442	10	2	Nil
357*	380	77	11	350	351	7	1	Nil

Note. Compare response of birds denoted by asterisk to combined fractions F and B and to fraction A (Table II).

Table II. *Vitamin B₃ effect of liver fractions B + F, and of fraction A.*

Bird	Max. wt. (g.)	Wts. before and after admin- stration of fractions		Duration of fractions B + F (days)	Gain in wt. (g.)	Av. daily gain in wt. (g.)	Wts. before and after administra- tion of fraction A		Duration of frac- tion A (days)	Gain in wt. (g.)	Av. daily gain in wt. (g.)
		B (g.)	F (g.)				(g.)				
A											
293	400	362	384	7	22	3.1	384	395	9	11	1.2
91	356	323	343	9	20	2.2	—	—	—	—	—
299	398	379	382	3	3	1.0	382	388	12	6	0.5
822	426	401	410	9	9	1.0	410	419	14	9	0.6
247	428	389	409	10	20	2.0	409	413	7	4	0.5
190	500	442	479	9	37	4.1	479	497	8	18	2.2
287	474	434	451	5	17	3.4	—	—	—	—	—
289	440	—	—	—	—	—	377	432	20	55	2.7
294	373	—	—	—	—	—	327	361	21	34	1.6
B											
248	500	—	—	—	—	—	475	498	8	23	2.8
296	360	—	—	—	—	—	345	363	4	18	4.5
719	398	—	—	—	—	—	378	399	6	21	3.5
733	500	444	468	7	24	3.4	468	474	26	6	0.2
357	380	351	380	8	29	3.6	—	—	—	—	—

Note. 1 bird failed to respond to liver fractions or subsequently to whole wheat.

definite effect was observed when the combined fractions F and B were given to a group of birds including 7 which had previously received independently one or other of these fractions. The results are recorded in Table II A. In certain cases, after the response to these fractions had been observed, they were substituted by fraction A, the administration of which was continued either until maximum weight was restored or until no further rise was produced. Two birds received fraction A without previous administration of B and F. It is seen that the responses to the combined fractions and to fraction A are similar to that previously recorded with crude liver preparations [Carter and O'Brien, 1935]. Seven birds rose to within 10 g. of their maximum weight. In birds 287 and 91 an initial response was observed but complete restoration occurred neither with

the liver supplements nor with whole wheat. In Table II B the effect of liver concentrates is seen in 5 birds which, having reached maximum weight on a vitamin B₁ concentrate and caseinogen, showed partial failure to maintain this weight when these supplements were continued for 40–60 days. Recovery was observed in four cases on addition of liver concentrates; one bird which did not respond to the supplement showed no recovery on wheat or mixed corn.

DISCUSSION.

The difficulty in securing a sufficient number of birds for testing our vitamin B₃ preparations has limited the data upon which our evidence is based. The results so far obtained however point to the presence in these preparations of one or possibly two factors which may be necessary for pigeon nutrition. No effect has been observed with fraction B alone. With fraction F, the flavin fraction, definite response has been observed in two or three cases, but it is much less definite than the effect of the two fractions combined. These facts may be explained either on the ground that the state of depletion of these factors may vary independently from bird to bird, or that the birds are already receiving a partial supply of the factor present in fraction B from other sources, for instance the vitamin B₁ concentrate. The evidence does not permit us to decide between these alternatives. Moreover, it is premature to suggest any possible relationship between the factors in our liver concentrates and (a) factors in liver described by other workers and (b) other components of the vitamin B complex. With regard to the former the presence in liver of a factor necessary for the normal nutrition of the rat [Mapson, 1932; Seegers and Smith, 1932], and of factors necessary for the chick [Hogan and Boucher, 1933; Elvehjem *et al.*, 1932–33; Kline *et al.*, 1934] have been reported. The last group of workers observed that young chicks on a diet including vitamins B₁ and B₂ failed to grow normally and developed a characteristic leg paralysis accompanied by degeneration of the cerebellum. Normal growth and protection from paralysis resulted from feeding preparations of liver. They concluded that the growing chick requires, in addition to vitamins B₁ and B₂, a growth factor insoluble in water and stable to autoclaving and an antiparalysis factor, soluble in water and destroyed by autoclaving, both being present in liver. Elvehjem and Koehn [1935] found that chick pellagra could be cured by a liver fraction from which flavin had been removed by adsorption on fuller's earth. This observation has been confirmed by Lepkovsky and Jukes [1935]. These workers found that the fuller's earth adsorbate of liver extract containing hepatoflavin markedly stimulated the growth of chicks. There is thus the possibility that the factors present in liver and necessary for the chick, namely a factor adsorbed on fuller's earth (? flavin) and one present in the filtrate after treatment with this reagent, are also required by the adult pigeon. It is possible that the activity of our fraction F may be ascribed to its flavin content but until further purification has been effected this cannot be definitely settled. We have suggested that fraction B makes adequate a supply of a factor present in the impure vitamin B₁ concentrate. This is believed to contain vitamin B₆ and is a source of vitamin B₅. At present, we are unable to define the relation between the factor in fraction B, vitamin B₅ and vitamin B₆. Nevertheless, our results give support to the view that a pigeon receiving a well-balanced basal diet requires in addition to vitamin B₁ at least two other factors.

SUMMARY.

1. A method is described for the preparation of a liver concentrate with vitamin B₃ activity.

2. From this concentrate two fractions have been obtained: (a) one adsorbed on fuller's earth carrying with it flavin and (b) one present in the filtrate after treatment with this reagent, fraction B. The flavin fraction showed some weight restorative potency whereas fraction B given alone was without effect. Greatest activity was shown when the two fractions were given together.

3. The relation of these results to the factors necessary for the nutrition of the chick is discussed.

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X. THE UTILISATION OF CO₂ IN THE DISSIMILATION OF GLYCEROL BY THE PROPIONIC ACID BACTERIA.

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REDTENBACHER [1846] was probably the first to study the fermentation of glycerol by the propionic acid bacteria. Largely by accident he obtained a good growth of these organisms in probably impure culture and found propionic acid as substantially the only product. It remained for Van Niel [1928] to confirm the results of Redtenbacher with pure cultures. He found that glycerol was converted quantitatively into propionic acid under anaerobic conditions with no formation of gas. Van Niel suggested that glyceraldehyde is an intermediate product which is reduced to propionic acid. Pett and Wynne [1933, 1] obtained preliminary evidence for the formation of methylglyoxal and of glyceraldehyde (or dihydroxyacetone) from sodium β -glycerophosphate by fermentation with dried propionic acid organisms. These authors indicate that their identifications are not conclusive. Wood and Werkman [1934] using both CaSO_3 and dimedon (dimethyldihydroresorcinol) as fixatives isolated and identified propaldehyde from fermentations of glycerol. They suggested that this compound occurs as an intermediary and is subsequently converted into propionic acid. Pyruvic acid has also been identified (unpublished results).

This brief review summarises our knowledge of the dissimilation of glycerol by the propionic acid bacteria. In the present communication it will be shown that propionic acid is not the only product which may be formed and that the dissimilation is more complex than found by previous investigators.

METHODS.

The volatile acids were determined by the partition method of Osburn *et al.* [1933]. Non-volatile acids were extracted with ethyl ether continuously for 12 hours from the residue of steam-distillations which was taken up in anhydrous Na_2SO_4 . The lactic acid was determined by the method of Friedemann and Kendall [1929]. The succinic acid was obtained by neutralising the hot solution with CaCO_3 , filtering and precipitating the calcium succinate in 85% ethyl alcohol. The calcium salt was determined by weight. This acid was also determined by the silver salt method [Moyle, 1924]. The original glycerol was determined by weight. The residual glycerol determination was made on an acidified aliquot part of the medium which was evaporated on a steam-bath to 50-75 ml., neutralised, taken up in plaster of Paris and extracted continuously with acetone for 8 hours. The acetone extract was reduced to 50-75 ml., distilled water added and the acetone removed by further distillation. The glycerol was determined in this preparation by the method of Wagenaar [1911]. The original

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CO₂ of the medium was calculated equivalent to the weighed quantity of CaCO₃ used in the medium. The liberated CO₂ was determined in two ways. It was either absorbed in soda-lime and weighed or it was absorbed in standard alkali and the excess alkali titrated with standard HCl after the addition of an excess of BaCl₂ to precipitate the CO₂. The soda-lime was used in a train of 6-inch U-tubes with CaCl₂ as the drying agent. When alkali was used it was contained in two bottles connected by a syphon. The first bottle was attached to the fermentation flask; the second was supplied with a soda-lime tube to prevent entrance of CO₂ from the air. The syphon permitted displacement of the alkali as gas was formed during fermentation. The alkali was transferred to a bottle fitted with a bubble spiral-absorber for the collection of residual CO₂. The residual CO₂ was obtained as follows. The fermentation flask was attached to a reflux condenser which led to the soda-lime train or bubble spiral-absorber and the CO₂ was liberated by adding a known volume of dilute H₂SO₄ slightly in excess of that necessary to react with the CaCO₃, a slow current of CO₂-free air was passed through and the contents finally brought to the boil. The CO₂ collected *minus* the CO₂ added as CaCO₃ equals the CO₂ produced by the fermentation. The method proved accurate with known quantities of CaCO₃.

The media used were as follows. The experiments of Table II were carried out with 700 ml. of medium consisting of glycerol 3%, CaCO₃ 2% and yeast extract (Difco) 0.4% in 1 litre Erlenmeyer flasks. These fermentations were maintained anaerobic by continuously bubbling oxygen-free nitrogen through the fermenting medium. The CO₂ was absorbed in soda-lime. The results shown in Table III were obtained with 800 ml. of medium containing glycerol 2%, CaCO₃ 1.14% and yeast extract 0.4%. Conditions were made anaerobic by displacing the air in and above the medium with nitrogen immediately after inoculation. The CO₂ was collected in alkali. Constituents of the media were sterilised separately at 20 lb. pressure for half an hour and mixed at the time of inoculation. 3 5-day cultures grown in a yeast extract medium with 0.5% glucose and equivalent to 5% by volume were used as inoculum. The flasks were shaken twice daily to aid the buffer action of the carbonate. Purity of cultures before and after fermentation was established by the Kopeloff-Gram stain, absence of growth on aerobic slants and the zone of growth and colony formation in agar shakes. Incubation was at 37°, for the fermentations of Table II and at 30° for those of Table III. The incubation period was 35 days.

The cultures are identified in Table I. Complete descriptions of the species are given by Werkman and Brown [1933].

EXPERIMENTAL.

Results are expressed as millimoles per litre of medium in Table II and per 100 millimoles of fermented glycerol in Table III. The carbon recovery (Table III) is calculated first on the basis of the glycerol fermented and CO₂ utilised and

Table I. *Identification of cultures.*

Culture no.	Species	Culture no. used by other investigators*
11 W	<i>P. petersonii</i>	<i>Sherman</i> —22, Van Niel—20
15 W	<i>P. technicum</i>	<i>Sherman</i> —10
34 W	<i>P. arabinosum</i>	<i>Hitchner</i> 61
49 W	<i>P. pentosaceum</i>	Foote, <i>Fred</i> and Peterson —11 Van Niel—4
52 W	<i>P. shermanii</i>	Foote, <i>Fred</i> and Peterson —19
4875	<i>P. pentosaceum</i>	American Type Culture Collection

* Culture received from investigator whose name is italicised.

Table II. *Dissimilation of glycerol by propionic acid bacteria.*

Culture no.	Products per litre			
	Propionic acid mM.	Acetic acid mM.	Succinic acid mM.	CO ₂ utilised mM.
4875	101.7	6.5	32.2	46.6
34 W	137.6	2.8	28.8	43.0
49 W	128.0	7.7	19.1	25.6
49 W	168.9	9.3	130.0	—

Table III. *Dissimilation of glycerol by propionic acid bacteria.*

Culture no.	Glycerol fermented per litre mM.	CO ₂ utilised per 100 mM. of fermented glycerol mM.	Products per 100 mM. of fermented glycerol			Carbon recovery		Oxidation-reduction index	
			Propionic acid mM.	Acetic acid mM.	Succinic acid† mM.	Basis-glycerol plus CO ₂ %	Basis-glycerol only %	Basis-glycerol plus CO ₂	Basis-glycerol only
49W	212.6	37.7	55.8	2.9	42.1	101.2	114.0	1.081	2.550
34W	209.0	43.2	59.3	2.0	34.5	93.1	106.6	0.925	2.270
52W*	112.0	20.0	78.1	5.9	8.7	94.6	101.0	0.918	1.386
11W†	218.4	1.1	89.3	2.6	3.9	96.5	96.8	1.135	1.162
15W	176.4	12.3	78.4	5.8	7.8	89.1	92.6	1.047	1.376

* 7.0 mM. of lactic acid produced per 100 mM. of fermented glycerol.

† 0.5 mM. of lactic acid produced per 100 mM. of fermented glycerol.

‡ Succinic acid identified by melting-point and mixed melting-point.

secondly on the basis of glycerol alone. The redox indices (Table III) are calculated on the same bases. The redox (oxidation-reduction) index of a fermentation is obtained by (1) multiplying the mM. of each product by its respective oxidation or reduction value based on water as zero (H₂ is equivalent to one atom of oxygen) [cf. Johnson *et al.*, 1931]; (2) dividing the sum of oxidation values by reduction values. A perfect balance is represented by an index of 1.0. Propionic acid has a reduction value of 1, succinic an oxidation value 1 and acetic acid is neutral. Glycerol has a reduction value of 1, therefore its utilisation is represented as an oxidation and the glycerol fermented is given an oxidation value 1. Likewise, the utilisation of CO₂, an oxidised compound, represents a reduction and its reduction value is 2.

The glycerol fermented was not determined in Table II. The quantity of non-reducing compounds was not sufficient to influence the fermentation balances.

DISCUSSION.

The most significant fact shown by the data is the apparent utilisation of CO₂ by the propionic acid bacteria. This was evident, since the CO₂ at the conclusion of the fermentation was not equivalent to that of the original medium in the form of CaCO₃. This observation has been substantiated by two types of calculation of especial value, *i.e.* carbon recovery and redox index. If CO₂ is utilised, and is in turn (after synthesis) dissimilated, then calculations based on the assumption that glycerol is the sole source of carbon, should show an excess of products, *i.e.* the calculated recovery of carbon will exceed 100%. Table III shows that this occurred and that calculations based on glycerol plus CO₂ are acceptable. The calculation of carbon recovery is not in all cases entirely satisfactory proof of CO₂ utilisation, but the oxidation-reduction balance is convincing. CO₂ contains but one carbon and requires a large utilisation to show a

detectable change in the carbon balance; in the oxidation-reduction balance the CO₂ is highly oxidised and therefore has a marked effect. The data show that results calculated on the basis of glycerol *plus* CO₂ are reasonable and acceptable. The fact that the chemical analysis shows a decrease of CO₂ is perhaps proof enough of CO₂ utilisation. However, the carbon and oxidation-reduction balances furnish additional evidence.

The CO₂ analysis has been checked by two different methods, and four species of *Propionibacterium* of known purity, each received from a different source, have shown a distinct utilisation of CO₂ under the conditions of our experiments. Every culture examined with the possible exception of 11W showed ability to utilise CO₂. This behaviour is probably characteristic of the genus *Propionibacterium*.

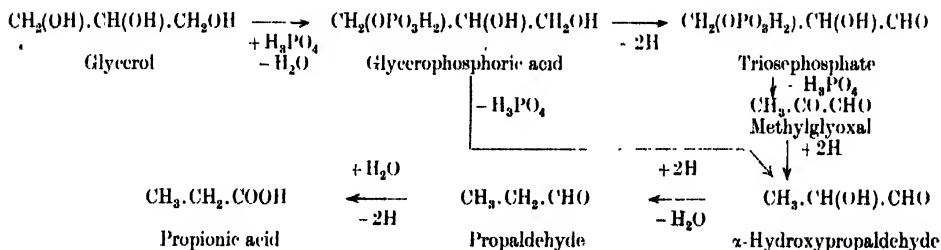
This observation requires a reinterpretation of previous results. Investigators have not considered the possibility of CO₂ utilisation in constructing schemes of dissimilation. If one considers the limited number of bacteria which have been shown to utilise CO₂ and also that such forms differ markedly from the propionic acid bacteria, failure to consider the possibility of CO₂ utilisation may be understood. It is of interest that a number of investigators have found that growth of bacteria in general is inhibited in the absence of CO₂. Rockwell and Highberger [1927] suggested that CO₂ is utilised by bacteria and expressed the opinion that it is the only direct source of carbon. It is possible that the utilisation of CO₂ as a source of carbon may not be limited to the small group of bacteria now recognised. Since most bacteria produce CO₂ during their growth it is difficult to determine whether they utilise CO₂. In the present case, the propionic acid bacteria utilised more CO₂ than was produced and thus offered direct evidence of CO₂ consumption.

One important problem, which requires consideration in relation to the data presented, is the mechanism of succinic acid formation. A number of investigators working particularly with yeast and fungi [Butkewitsch and Federoff, 1930; Wieland and Sonderhoff, 1933] have reported that succinic acid is formed by a condensation of two molecules of acetic acid. Virtanen [1925; 1934] and Virtanen and Karstrom [1931], however, have suggested that the propionic acid bacteria produce succinic acid from glucose by a 4- and 2-carbon cleavage of the hexose molecule. Virtanen's proposal was prompted by the observation that the propionic acid bacteria in the presence of toluene form succinic and acetic acids from glucose with no gas. This observation appeared incompatible with schemes involving a 3-carbon cleavage and the formation of 2-carbon compounds by a 2- and 1-carbon cleavage. The absence of CO₂ or other 1-carbon compounds appeared conclusive proof against such a scheme. However, the present evidence of the utilisation of CO₂ by the propionic acid bacteria leaves no reason to assume that the 1-carbon compounds should equal the sum of the 2-carbon compounds. It is necessary in the light of our present knowledge to leave open the possibility of a 4- and 2-carbon cleavage.

The mechanism of fermentation (Tables II and III) is of interest but at present must remain largely speculative. The scheme of Van Niel is not complete since it does not include succinic acid. His suggestion that the succinic acid is formed from the yeast extract is excluded in these fermentations as in some a quantity of succinic acid was obtained which exceeded the total quantity of yeast extract used in the medium. The formation of the 4-carbon compound (succinic acid) from the 3-carbon compound (glycerol) is proof of synthesis. It is possible that the synthesis yields a 6-carbon compound which is dissimilated to succinic acid by a 4- and 2-carbon cleavage. However, such a scheme for the

glycerol fermentation would require a synthesis from a 2-carbon compound since the 2-carbon and 4-carbon compounds do not occur in equivalent quantities. It seems more probable that part of the glycerol is dissimilated to 2- and 1-carbon compounds and that the 1-carbon compound is completely utilised in the synthesis of a compound which is subsequently fermented whilst the 2-carbon compound, probably acetic acid, condenses to yield succinic acid. The evidence presented justifies the assumptions relative to the utilisation of the 1-carbon compound. With regard to the formation of succinic acid by a condensation of 2-carbon compounds more complete evidence will be presented elsewhere. The results obtained by different methods indicate that the propionic acid bacteria can produce succinic acid by a condensation of 2-carbon compounds and it is probable that the condensation involves acetic acid. The formation of succinic acid from glycerol is indirect support of such a mechanism although the possibility cannot be disregarded that other reactions are occurring. It is possible that the succinic acid is formed in more than one way.

Explanation of the formation of propionic acid also offers difficulties. Wood and Werkman [1934] furnished some information by their isolation of propaldehyde but the question arises as to the manner of formation of the propaldehyde. The investigations of Embden *et al.* [1933] and Meyerhof and Kiessling [1933] have emphasised the importance of glycerophosphoric acid and phosphoglyceric acid in the carbohydrate metabolism of yeast and muscle tissue. This suggests the possible rôle of these compounds in the dissimilation of glycerol by the propionic acid bacteria. In the schemes proposed by Embden and Meyerhof the triosephosphate is converted into phosphoglyceric acid. To obtain propaldehyde following such a conversion the carboxyl group would have to be reduced to a carbonyl group. It is doubtful whether the propionic acid bacteria can bring about such a conversion. A more probable series of reactions leading to the formation of propaldehyde and propionic acid is that shown below.



Mechanism of the dissimilation of glycerol to propionaldehyde and propionic acid.

Pett and Wynne [1933, 2] have found that the propionic acid bacteria can dephosphorylate glycerophosphoric acid and also [1933, 1] obtained preliminary evidence of the occurrence of methylglyoxal and glyceraldehyde (or dihydroxyacetone). Reduction of methylglyoxal to propaldehyde might reasonably be expected, for the reduction of pyruvic acid to propionic acid is readily accomplished by these organisms. The proposed scheme involving methylglyoxal appears from our present knowledge to be a logical means of obtaining propaldehyde although other reactions cannot be excluded. For example, the glycerophosphoric acid may go directly to α -hydroxypropaldehyde.

Phosphoglyceric acid may not play a rôle in the formation of propaldehyde although it may be an important intermediary in the fermentation yielding pyruvic acid and subsequently acetic acid, CO_2 and propionic acid. There is no

reason apparent why methylglyoxal and phosphoglyceric acid cannot occur in the same fermentation or why a compound cannot be formed by more than one series of reactions.

It is not clear why our fermentations gave results so different from those of Van Niel [1928]. His data in which CaCO₃ was used as a buffer give no information relative to the CO₂ but show that the dissimilation was not the same, with the possible exception of that obtained with culture 11W. Van Niel accounted quantitatively for his fermented glycerol as propionic acid. The action of CO₂ as a hydrogen acceptor is made evident in our fermentations by the production of the oxidised product succinic acid. It is apparent that a large CO₂ utilisation is accompanied by a greater production of succinic acid or some other oxidised compound.

SUMMARY.

An investigation has been made of the dissimilation of glycerol by bacteria of the genus *Propionibacterium* and the products have been determined quantitatively. The results obtained support the following conclusions.

1. CO₂ obtained from CaCO₃ is utilised by the propionic acid bacteria in their fermentation of glycerol.
2. Propionic, acetic, succinic and occasionally lactic acids are produced from the glycerol. CO₂ acts as a hydrogen acceptor permitting the formation of oxidised products.
3. The utilisation of CO₂ by the propionic acid bacteria necessitates further interpretation of data and places in question the evidence for the 4- and 2-carbon cleavage.
4. The formation of succinic acid from glycerol offers indirect evidence of its formation by a condensation of 2-carbon compounds.
5. The mechanism of glycerol dissimilation is shown to be complex, involving synthetic reactions with subsequent fermentation of a synthesised product.

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XI. POLYPLOIDY AND VITAMIN C.

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(Received November 25th, 1935.)

As a result of some observations made on the relationship between vitamin C and polyploidy in apples [Crane and Zilva, 1931: 1932] we took the opportunity a few years ago [Sansome and Zilva, 1933] to extend this investigation to the tomato, a fruit which is much more amenable to the induction of polyploidy. The results obtained in our inquiry strengthened in a clear-cut fashion the view previously indicated by the experiments on apples, that polyploidy is associated with a higher vitamin C content of the fruit. The antiscorbutic activity of the tetraploid tomatoes was found to be double that of the diploid fruits. Furthermore, this observation held true in tetraploids derived from diploid stock of the genetic constitutions **DOPR**, **dopr**, **DOPr** and **dopR**. The possibility of other factors exercising an influence in this connection was, however, pointed out at the time. In pursuing the investigation we found that this was indeed the case; in fact, in the crops of the following years the disparity in the vitamin contents of the two forms was much less than that found originally (unpublished work). The evidence we have so far obtained does not enable us to draw conclusions, but reference to the subject in a recent publication by McHenry and Graham [1935] calls for a few remarks.

These workers, determining the ascorbic acid by indophenol titration in comparable fruits belonging to tetraploid and diploid forms, found that the latter tomatoes contained about 84 % of the ascorbic acid found in the former, a difference considerably less than that recorded in our published work. They further found that small tomatoes possessed a higher concentration of ascorbic acid than the larger fruits and suggested that part at least of the differences found by us was due to the differences in the sizes of the two forms of the fruits. We are of the opinion that the size of the fruit, in the range used in our experiments, even if it were to have a bearing on the vitamin C concentration, is not a factor of any significance controlling the difference in concentration of ascorbic acid in the two forms of the fruit.

In our original communication we have already pointed out in a general statement that larger fruits did not show a lower concentration of ascorbic acid than did smaller fruits in the same form. We now take the opportunity of giving more details of this observation. The experiments in question were performed in 1933. As pointed out by us then, the tomatoes were collected twice weekly, and in Table I are given the average weights of the tomatoes of each collection and the average ascorbic acid content per 100 ml. of juice (determined daily) for each collection. In these experiments the tetraploid were much smaller than the corresponding diploid tomatoes. It will, nevertheless, be observed that

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Table I.

	dRop		drop		DROP		DrOP		
	Average wt. of fruit g.	Average ascorbic acid con- tent per 100 ml. juice mg.	Average wt. of fruit g.	Average ascorbic acid con- tent per 100 ml. juice mg.	Average wt. of fruit g.	Average ascorbic acid con- tent per 100 ml. juice mg.	Average wt. of fruit g.	Average ascorbic acid con- tent per 100 ml. juice mg.	
Tetraploid	20	25.0	20	32.9	20	38.6	19	39.9	
	20	36.4	19	40.4	20	40.8	18	37.3	
	20	32.0	18	37.3	18	45.6	14	41.7	
	17	39.5	14	41.7	18	43.9	12	39.5	
	15	44.8	12	39.5	17	40.8	12	27.6	
	14	42.5	12	29.8	15	48.2	12	33.3	
	14	32.9	12	33.8	15	39.5	10	38.6	
	12	32.9	10	39.5	15	35.1	10	35.1	
	11	43.0	10	35.1	15	27.2	10	35.1	
	10	39.5	10	35.1	15	26.3	9	38.2	
	10	35.0	9	38.6	15	29.4	8	38.2	
	10	32.9	8	35.1	12	43.9	8	43.9	
	10	30.7	8	35.1	—	—	8	34.2	
	7	39.5	8	34.2	—	—	—	—	
Average	13.6	36.2	12.1	36.3	16.2	38.3	11.5	37.1	
Diploid	65	17.5	60	24.1	90	19.7	51	18.4	
	60?	15.8	50	22.8	62	19.7	46	24.1	
	55	21.1	48	23.2	60	21.0	44	19.3	
	55	20.6	44	29.4	55	20.6	40	24.1	
	50?	14.0	40	17.5	55	25.0	40	24.6	
	44	18.0	40	30.7	52	15.4	40	26.3	
	42	18.4	40	22.8	48	21.9	40	24.1	
	40?	15.1	40	19.7	40	22.8	36	24.1	
	40?	20.6	35	18.9	35	24.1	35	19.7	
	40?	14.5	32	26.3	30	23.2	30	19.7	
	40	13.2	30	26.3	30	17.5	30	38.2	
	40	26.3	30	21.9	27	17.5	30	24.1	
	25	28.5	30	21.0	25	36.4	—	—	
	25	25.4	22	15.4	—	—	—	—	
	Average	44.4	19.4	38.6	22.9	46.8	21.9	38.3	23.6

? Slight doubt exists as to the absolute accuracy of these weights.

Table II. DROP.

Tetraploid			Diploid		
Wt. of fruit	Diameter	Ascorbic acid content per 100 ml. juice	Wt. of fruit	Diameter	Ascorbic acid content per 100 ml. juice
g.	mm.	mg.	g.	mm.	mg.
77	57	15.4	70	50	21.9
40	44	20.6	70	50	21.9
36	38	31.1	42	38	21.0
32	32	25.9	26	25	24.6
30	38	27.2	26	38	17.5
29	32	34.2	24	25	15.8
29	32	22.4	23	38	27.2
27	32	21.5	21	32	19.3
24	38	45.6	15	25	26.8
21	32	24.6	9	13	
19	32	26.8	10	19	28.5
15	25		13	25	
Average	31.6	26.8	29.1	31.5	22.4

there is no evidence that the ascorbic acid content is conditioned within any form by the size of the fruit. Similar evidence was obtained by us during 1934 and 1935. In Table II we give the results recorded in single fruits. This experiment was performed with the special purpose of testing McHenry and Graham's suggestion. Unfortunately, owing to the fact that their communication came to our notice early in October, we could only utilise fruits gathered at the end of the 1935 season, a circumstance which limited our scope. These figures also do not seem to suggest that the size of the tomato has any bearing on the anti-scorbutic potency of its juice.

In continuing our investigation during 1934 and 1935 we were struck by the fact that we could not record again such a great inequality between the ascorbic acid contents of the tetraploid and diploid forms as that observed in 1933. In the latter experiments, the evidence was clear-cut and, as already mentioned, the disparity found was of the same high order in four sets of tomatoes of different genetic constitution. It therefore seems to us that an unknown factor or factors enter into the scheme which is capable of modifying the unequal content of ascorbic acid in the two forms. We were unable so far to obtain any definite evidence on this point, being greatly handicapped by the facts that this work can only be carried out during a short period of the year and that in planning the test experiments the conditions can only be partially controlled by the worker who is otherwise greatly dependent on the elements. We hope, however, that in time favourable conditions may aid this search.

It is appropriate to mention here that all the titrimetric estimations of ascorbic acid with the exception of those contained in Table II have been checked by biological tests.

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XII. THE ISOLATION AND IDENTIFICATION OF A COMBINED FORM OF OESTRIOL IN HUMAN PREGNANCY URINE.

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In the past scant attention has been paid to the problem of the chemical nature of the "combined" ether-insoluble forms of the oestrogenic substances which are present in human and equine pregnancy urines and which yield the free hormones by acid hydrolysis. Two years ago the present authors planned a research which, it was hoped, would culminate in the isolation and chemical identification of these compounds. At the outset it was realised that slow progress would be made if only biological methods of assaying oestrogenic potency were available. Efforts were, therefore, first directed towards devising a convenient and accurate chemical method of assay. Fortunately, Kober [1931] had previously described a colour reaction for oestrin which, after the introduction of certain modifications, was found suitable for the purpose. Using such a modified Kober test, a method of purification and fractionation of pregnancy urine extracts was elaborated which allowed separate determinations of oestrone and oestriol to be made with a reasonable degree of accuracy [Cohen and Marrian, 1934]. A study was then made [Cohen and Marrian, 1935] of the factors influencing the hydrolysis of the combined forms of the two hormones. This made possible the accurate assay of the oestrone and oestriol present in human pregnancy urine and also provided evidence regarding the stability of the compounds so that methods for their purification could be more readily devised.

In the present communication are reported the results of attempts made to isolate and identify the combined form of oestriol present in human pregnancy urine. Since the amounts of oestrone present in human urine are relatively so small, it was felt that an attempt to isolate the combined form of this compound from the same source would at present be unprofitable.

Methods of concentration.

In 1932 experiments (unpublished) were carried out by one of us (G. F. M.) in collaboration with Mr E. R. Smith which suggested that the "combined" oestrin could be extracted from evaporated urine residues by ethyl alcohol or from untreated urine by butyl alcohol. Owing to the difficulties of biological assay the results were not entirely conclusive and the work was not continued at that time. In the present work it has been found that the combined oestrone and oestriol can be almost quantitatively extracted from urine by both these methods. For the initial process extraction with butyl alcohol was preferred since it eliminated the time-consuming necessity of evaporating the urine to dryness.

In order to elaborate methods of further purification of the butyl alcohol extracts, it was found helpful to make an assumption about the probable nature of the oestriol complex. It seemed reasonable to suppose that the large water-insoluble oestriol molecule would be held in aqueous solution in the urine by

combination as an ester with some polybasic acid. Although subsequent work failed to prove the correctness of this hypothesis, its temporary adoption was justified since it enabled a satisfactory method of isolation to be devised.

Assuming the acidic nature of the complex, it was thought desirable to acidify the urine slightly before extraction with butyl alcohol, since it seemed probable that salts of the complex would be less soluble in butyl alcohol than the free acid. This procedure involves no risk of loss of the complex since it was previously shown [Cohen and Marrian, 1935] that little hydrolysis of combined oestrin occurs even at p_{H} 1.0 at low temperatures. The belief that the complex possessed a free acidic group was confirmed by the discovery that it could be removed from the butyl alcohol by extraction with alkaline solutions, preferably *N*/10 sodium hydroxide. By acidification of such alkaline extracts, extraction with butyl alcohol and evaporation of the latter, concentrates containing most of the combined oestriol originally present in the urine and almost free from urea were obtained. Extraction of such concentrates with 90% ethyl alcohol removed most of the complex leaving behind considerable quantities of inorganic salts.

The evidently acidic nature of the oestriol complex suggested the use of an organic base such as pyridine as a possible means of further purification. It was found that whilst dry pyridine would not readily remove the complex from the product obtained by the evaporation of the 90% alcoholic extract, pyridine containing small amounts of water was extremely effective: 92% aqueous pyridine proved to be the most suitable mixture for the purpose. This pyridine-soluble material was found by colorimetric assay to contain about 0.5-0.6% of oestriol.

Advantage was now taken of the low solubility of the oestriol complex in anhydrous pyridine for devising a method of removing the material from the pyridine solution, thus obviating the tiresome necessity of low temperature evaporation of the pyridine extract. The addition of a suitable amount of benzene to the pyridine solution caused an aqueous fraction to separate, and when this mixture was shaken with water the oestriol complex was quantitatively transferred to the aqueous phase. At the same time a further purification was effected in so far as about 25% of the original solids, including all the free oestriol, were left in the pyridine-benzene layer.

Extraction of the aqueous solution of the complex with a water-immiscible nitrogenous base now suggested itself. It was found that the complex could be readily removed from the aqueous extract by shaking with quinoline. Owing to the difficulty in evaporating the quinoline no data concerning the degree of purification effected were obtained, but since the aqueous phase remained highly pigmented, it was clear that some impurities were being removed. For the removal of the oestriol complex from the quinoline solution a process similar to that used in the pyridine process was impracticable since the solubility of the complex in quinoline is apparently independent of the presence of water. Attempts were made to remove the quinoline as its water-soluble hydrochloride by adding the extract to a large volume of butyl alcohol and washing the mixture with dilute hydrochloric acid. This process, however, resulted in a considerable loss of the oestriol complex and was therefore abandoned. Extraction of the quinoline with aqueous alkalis fortunately proved to be more effective. By shaking the quinoline solution with 10% aqueous sodium carbonate traces only of the complex but much pigmented material were transferred to the aqueous phase. On the other hand, extraction with *N*/10 sodium hydroxide removed over 80% of the complex. The procedure was therefore finally adopted

of extracting the complex from the quinoline with $N/10$ sodium hydroxide after preliminary washing with 10% sodium carbonate. The complex was separated from inorganic matter introduced during this purification by slight acidification of the alkaline extract and subsequent extraction with butyl alcohol. Assays on this alcoholic extract showed that the whole quinoline process had resulted in a 10-fold degree of purification with only 30% loss of the total combined oestriol obtained from the preceding pyridine process.

The success attending extraction of the quinoline solution with aqueous solutions of varying degrees of alkalinity, suggested that it might be profitable to explore more fully the possibilities of a similar alkali fractionation of butyl alcohol solutions. A series of experiments conducted towards this end resulted in the adoption of the following process. The butyl alcoholic solution of the material obtained from the quinoline process was first washed with 0.8% sodium carbonate solution, thereby effecting the removal of much pigmented material but of only small amounts of the oestriol complex. The complex was subsequently removed from the butyl alcohol by extraction with $N/50$ sodium hydroxide. Inorganic material introduced by this process was separated in the usual manner by transference of the combined oestriol to butyl alcohol. This process resulted in about a 4-fold purification with only 12% loss in the total combined oestriol. At this stage the product contained about 15%¹ of oestriol.

In an attempt to devise methods of further purification the solubilities of this material in various organic solvents were studied. Cold acetone extracted the combined oestriol quantitatively, leaving behind a brown oil which amounted to about 20% by weight of the original material. Attempts to purify further the acetone-soluble material by the addition of solvents such as benzene and chloroform, in which the complex is insoluble, were ineffective.

It was thought that at this stage it might be profitable to attempt the separation and purification of a metallic salt of the complex. Alcoholic potassium hydroxide was found to precipitate a heavy greyish-white material from an alcoholic solution of the acetone-soluble fraction. This precipitate contained about 85% of the combined oestriol present. Since the supernatant liquid was deeply pigmented, it was clear that a considerable degree of purification had been effected.

On adding dilute aqueous barium chloride to an aqueous solution of the crude potassium salt, a light yellow precipitate was thrown down. The supernatant liquid was again deeply pigmented and was found to contain little combined oestriol. On treatment of the barium salt with hot dilute hydrochloric acid and filtering while hot, a solution was obtained which on cooling deposited a nearly white amorphous solid. A second precipitation from hot water yielded a material which contained about 50% by weight of oestriol.

Nature of the final product.

This material, although not crystalline, contained such a high proportion of oestriol that it obviously represented a highly concentrated preparation of the sought-for complex. The substance melted at 193–197° with decomposition after preliminary sintering at 180°. Only 46 mg. of this final white precipitate being available it was decided to investigate its properties and chemical nature without any further attempts at purification.

¹ As later pointed out the method of oestrum assay employed on early concentrates gave values which, although relative, could not be regarded as strictly quantitative. They did, however, supply a working basis and hence are reported, even though subsequent work showed them to be 25–50% too low.

Sulphur, halogens, phosphorus and nitrogen were absent. Since many phenolic substances are excreted from the body in conjugation with glucuronic acid, the possibility was entertained that the complex might be a derivative of this acid. The fact that it decomposed at its melting-point with the evolution of gas lent some support to this idea. As the amounts of material were so small it was not considered advisable to try to isolate glucuronic acid or one of its easily identifiable derivatives from the products of acid hydrolysis of the complex. Since, however, the Tollens naphthoresorcinol test was strongly positive on as little as 0.5 mg. it seemed very probable that the material was indeed a glucuronic acid derivative.

Carbon and hydrogen determinations gave figures closely in agreement with those required for a substance of the formula $C_{24}H_{34}O_6$. An oestriol glucuronic acid complex would have the formula $C_{24}H_{32}O_6$; there can, therefore, be no reasonable doubt concerning the nature of the isolated substance. Further evidence was obtained when the barium salt of the complex was found to have a barium content close to that required for the barium salt of an oestriol glucuronic acid.

The substance did not reduce Benedict's solution indicating that the conjugation is through the terminal aldehyde group of the glucuronic acid. After hydrolysis with dilute hydrochloric acid the reduction test was strongly positive. Millon's test was strongly positive in the cold, which may perhaps indicate that the phenolic hydroxyl group of the oestriol is free. The possibility was borne in mind that the nitric acid present in the reagent might have caused sufficient hydrolysis to render the test positive even though the phenolic group was originally masked in the compound. The fact that salicin, under the same conditions, gave a completely negative Millon test lent no support to this idea.

The questions of the physiological activity of this interesting derivative of oestriol and of its possible relationship to the placental principle "emmenin" [*cf.* Collip *et al.*, 1934] will be dealt with in subsequent communications.

EXPERIMENTAL.

Method of assay.

The amounts of combined oestriol present in the concentrates at different stages of the purification process were determined colorimetrically by the method previously described [Cohen and Marrian, 1934]. The conditions of acid hydrolysis previously found to be satisfactory for fresh urine [Cohen and Marrian, 1935], however, had to be altered owing to the relative or complete absence of urea from the concentrates. In general the following procedure was finally adopted: an aliquot portion of the concentrate, estimated roughly to contain about 1 mg. of oestriol, was diluted to 100 ml. with water, acidified with HCl to p_H 1.0 and, after the addition of a further 0.5 ml. of concentrated acid, heated in the autoclave at 15 lb. pressure for $1\frac{1}{2}$ hours. The free oestriol in the hydrolysis mixture was estimated in the usual manner.

Although no great confidence was felt that this method of hydrolysis gave a quantitative yield of oestriol from its combined form for all urine concentrates, the results were of comparative value and were sufficiently accurate to make possible the elaboration of a method of purification. As will be shown later, it was found possible to carry out direct colorimetric assays without previous hydrolysis on certain of the more highly purified concentrates obtained in the later stages of the process.

Collection and extraction of urine.

100 l. of fresh urine, from women in the 8th and 9th months of pregnancy, were collected in bottles containing toluene as a preservative and evaporated in a vacuum still to about one-fifth of the original volume. When it was not possible to concentrate the urine immediately after collection, it was stored at 0° in order to minimise bacterial hydrolysis of the combined oestriol. The concentrate was acidified to p_{H} 2.0 with HCl, saturated with NaCl and then extracted six times with butyl alcohol, the total volume of the latter used being about equal to that of the concentrated urine. The saturation with NaCl facilitated the breaking of the troublesome emulsions and furthermore by lowering the solubility of the butyl alcohol in the aqueous phase effected a considerable economy in the quantity of the solvent used.

Extraction of the combined oestriol from butyl alcohol by aqueous alkalis.

The following preliminary experiment was carried out. Four 100 ml. portions of the butyl alcoholic extract of a urine concentrate, each containing 1.3 mg. of oestriol in the combined form (by colorimetric assay), were extracted twice with 50 ml. volumes of (a) 10% Na_2CO_3 , (b) 25% Na_2CO_3 , (c) $N/10$ NaOH and (d) N NaOH respectively. The alkaline extracts were each assayed for total oestriol after acid hydrolysis and were shown to contain (a) 0.750 mg., (b) 0.325 mg., (c) 1.250 mg. and (d) 1.280 mg. of oestriol respectively.

The butyl alcoholic extract after careful neutralisation was therefore extracted six times with $N/10$ NaOH, the total volume of the latter equalling that of the original extract. Approximately 75% of the total oestriol present in the butyl alcohol was thus removed. In order to obtain a residue concentrate the alkali extract was acidified to p_{H} 2.0 with HCl, saturated with NaCl and extracted six times with butyl alcohol. This extract was washed with a small volume of saturated NaCl (acidified to p_{H} 2.0) to remove traces of urea that might have come through, then made slightly alkaline,¹ and evaporated to dryness under reduced pressure.

Extraction of the combined oestriol by aqueous alcohol.

Preliminary experiments showed that from such concentrates, 97% ethyl alcohol, slightly acidified to neutralise the free alkali in the concentrate, would only extract about 10% of the combined oestriol present. On the other hand, acidified 90% alcohol extracted over 80% of this material. Unacidified 90% alcohol extracted only about 40% of the combined oestriol present, clearly demonstrating the lower solubility of the alkali salt of the complex. The butyl alcohol residue was therefore shaken vigorously four times with 90% ethyl alcohol containing sufficient acid to bring the p_{H} of the suspension to about 5.0. It was found advantageous to add some fine sand to the flask to facilitate the separation of the solid from the sides. A total volume of 90% ethyl alcohol equal to about one-eighth of the volume of original concentrated urine was used. The alcoholic extracts were decanted from the insoluble solid, cleared by centrifuging, neutralised and evaporated to dryness under reduced pressure. Assays showed that only traces of oestriol remained in the alcohol-insoluble fraction.

¹ Wherever possible all solutions were made slightly alkaline before evaporation. As the stability of the complex appears to be greater at alkaline reactions losses due to slow hydrolysis have been minimised in this way.

Extraction with pyridine.

In order to determine the most effective pyridine solution for removing the oestriol complex from the 90 % ethyl alcoholic residue three samples, each containing 1.20 mg. total oestriol, were extracted with pyridine solutions containing different proportions of water. Subsequent analysis showed that a 90 % aqueous pyridine extract contained 1.15 mg., a 95 % extract contained 0.85 mg. and a 98 % extract contained 0.44 mg. of total oestriol. 92 % aqueous pyridine was found to be just as effective for the purpose as the 90 % solution and had the added advantage of removing less of the total solids.

The dry 90 % ethyl alcoholic residue was therefore extracted six times with 92 % aqueous pyridine, a total volume of the pyridine solution equal to one-eighth of the original volume of concentrated urine being used. The pyridine extracts were separated from the insoluble material by centrifuging and combined. Assays showed that about 16 % of the total oestriol remained in the pyridine-insoluble material. Evaporation of an aliquot portion of the pyridine extract and colorimetric assay of the residue after hydrolysis showed that the total weight of the pyridine-soluble material was 126 g. and that it contained 0.421 g. of total oestriol.

Distribution of pyridine extract between benzene and water.

To the pyridine extract were added two volumes of benzene and one of water. These proportions had been found optimum with respect to purifications effected, to completeness of removal of the combined oestriol and to minimising the amount of pyridine carried into the aqueous phase. After shaking, the aqueous layer was run off and washed once with benzene to remove final traces of pyridine. Subsequent assays showed it to contain about 75 % of the total oestriol originally present. Only free oestriol remained in the benzene-pyridine phase.

Distribution between quinoline and sodium carbonate and hydroxide solutions.

The aqueous solution containing the oestriol complex obtained by the pyridine purification process was extracted twice with quinoline, the total volume of the latter being about half that of the aqueous solution. Colorimetric assay showed that only traces of oestriol remained in the aqueous phase.

In a preliminary experiment three equal portions of this quinoline extract, each containing 1.40 mg. of oestriol, were extracted once with an equal volume of (a) 10 % Na_2CO_3 , (b) $N/10$ NaOH and (c) N NaOH solutions. Colorimetric assays carried out on these alkali extracts showed that they contained 0.030 mg., 1.260 mg. and 1.390 mg. respectively.

The following procedure was therefore adopted for the main bulk of the quinoline extract. The quinoline was first washed twice with 10 % Na_2CO_3 and then extracted twice with $N/10$ NaOH. In each case the total volumes of Na_2CO_3 and NaOH used were approximately equal to that of the quinoline solution. The combined oestriol was recovered from the NaOH solution by acidification to p_{H} 5.0 and extraction with butyl alcohol. An aliquot portion of the butyl alcoholic extract was washed with dilute HCl and then with water, evaporated to dryness, weighed and assayed for total oestriol. In this way it was determined that the total butyl alcoholic extract contained 5.9 g. of solids, of which approximately 0.25 g. was oestriol in a combined form.

Distribution between butyl alcohol and sodium carbonate and hydroxide solutions.

Several equal portions of the butyl alcoholic extract, each containing 0.7 mg. of combined oestriol in 5 ml. were extracted once with 5 ml. of a dilute alkaline solution. Colorimetric assays after hydrolysis showed that whereas 1% Na_2CO_3 extracted only 0.115 mg. of combined oestriol, $N/10$ NaOH removed 0.445 mg.

The remainder of the butyl alcoholic extract (containing about 200 mg. oestriol), after washing with dilute HCl to remove traces of quinoline and then with water, was further washed several times with a total equal volume of 0.8% Na_2CO_3 and then extracted with $N/50$ NaOH . These concentrations of carbonate and hydroxide tended to minimise losses of the complex. Assay showed that 48 mg. of oestriol nearly all of which was in the free state remained in the butyl alcohol.

The combined oestriol was recovered from the NaOH in the usual manner by extraction with butyl alcohol after acidification to p_{H} 5.0. The butyl alcoholic extract was washed with water and evaporated to dryness. This material weighed 1.17 g. and by colorimetric assay after hydrolysis was shown to contain 0.133 g. of combined oestriol. It was found that this material gave, without preliminary hydrolysis, apparently satisfactory direct colorimetric assays. Such an assay showed 0.300 g. of combined oestriol to be present. It would seem possible that either the hydrolysis was incomplete or that during the hydrolysis extensive destruction of the liberated oestriol occurred.

Precipitation of the potassium salt.

Extraction of this material with cold acetone left 0.22 g. of a brown oil which contained no oestriol. The residue obtained by evaporation of the acetone solution was dissolved in the smallest possible volume of ethyl alcohol and to this solution was added saturated ethyl alcoholic KOH until precipitation was complete. This precipitate was collected by centrifuging and washed once with a small volume of ethyl alcohol. Direct colorimetric assay showed that 50 mg. of combined oestriol were lost in the supernatant liquid and washing.

Precipitation and decomposition of the barium salt.

The crude washed potassium salt was dissolved in a small volume of hot water and to this was added $N/10$ BaCl_2 until precipitation was complete. After cooling, the barium salt was centrifuged off and washed first with a little cold water and then with ethyl alcohol. 12 mg. of oestriol were lost in the supernatant liquid and washings.

The crude barium salt was suspended in a small volume of hot water and treated with dilute HCl drop by drop until all but a few dark-coloured particles had gone into solution. The hot solution was filtered: the filtrate on cooling deposited a white amorphous precipitate. This material was collected by centrifuging and further purified by dissolving in hot water, filtering and cooling. The final product was dried *in vacuo* over calcium chloride and weighed 46.3 mg. It melted at $193\text{--}197^\circ$ with evolution of gas after sintering at 180° .

By working over the mother-liquors from the potassium and barium salt precipitations, 33 mg. more of material melting at about 170° were obtained.

Properties and chemical nature of the final product.

The material was soluble in hot water but less soluble in cold. It was precipitated from cold aqueous solution by half-saturation with ammonium sulphate. It was soluble in acetone, alcohol and ethyl acetate, and insoluble in ether, benzene and chloroform.

Qualitative tests by fusion with sodium showed that nitrogen, sulphur and halogens were absent. After decomposition with hot sulphuric and nitric acids no positive Briggs test for phosphorus was obtained.

A strongly positive naphthoresorcinol test (Tollens) was given by 0.46 mg. suspended in 1 ml. of water. 0.6 mg. failed to cause any visible reduction of 0.5 ml. of Benedict's reagent. 4.1 mg. were hydrolysed by heating with 3 ml. of $N/10$ HCl for 2 hours in an autoclave at 15 lb. pressure. After neutralisation with sodium bicarbonate the solution strongly reduced Benedict's reagent. Millon's reaction was strongly positive in the cold.

(Found: C, 61.60, 62.09; H, 7.19, 7.44 %. $C_{24}H_{31}O_9$ requires C, 61.76; H, 7.35 %. $C_{24}H_{32}O_9$ (oestriolglucuronic acid) requires C, 62.03; H, 6.95 %.)

The samples for combustion were dried over phosphorus pentoxide at 80 *in vacuo* and lost 4.95 % and 5.43 % of their weights respectively.

Theoretical loss of weight for $C_{24}H_{32}O_9$, $1.5 H_2O = 5.6$ %.

Barium salt. 10 mg. of the product were dissolved in 10 ml. of hot water. An equal volume of $N/10$ $BaCl_2$ was added and the white precipitate which formed on cooling the mixture was centrifuged. The precipitate was heated with 10 ml. of hot 50 % ethyl alcohol and the solid matter filtered from a small amount of insoluble material and cooled. The amorphous precipitate was centrifuged, washed with 0.5 ml. of cold water and dried *in vacuo* over $CaCl_2$.

(Found: Ba, 11.2 %. $(C_{24}H_{31}O_9)_2 Ba$ requires Ba, 12.9 %.)¹

Oestriol content. Direct colorimetric assays on 0.0652, 0.0931 and 0.1304 mg. gave figures of 52 %, 53 % and 49 % oestriol respectively. Calculated for $C_{24}H_{32}O_9$, $1.5 H_2O$, 58.6 % oestriol.

Six samples of 0.243 mg. of the product were hydrolysed for 0, $\frac{1}{2}$, 1, 2, 3 and 4 hours respectively with 50 ml. of $N/10$ HCl saturated with nitrogen (to minimise loss by oxidative destruction) in the autoclave at 15 lb. pressure. Each solution was made alkaline with 10 ml. of 10 % Na_2CO_3 and extracted with ether. Colorimetric assays were carried out on the residues obtained by evaporation of the water-washed ethereal solutions in the usual manner. The results are shown in the following table:

Time of hydrolysis hours	Oestriol determined mg.
0.0	0.019
0.5	0.049
1.0	0.052
2.0	0.092
3.0	0.083
4.0	0.0815

The maximum hydrolysis of 2 hours gave a figure of 38 % as the oestriol content of the compound.

SUMMARY.

A method is described by which a water-soluble, ether-insoluble non-crystalline substance containing approximately 50 % by weight of oestriol may be isolated from human pregnancy urine. The substance gives a strong naphthoresorcinol test for glucuronic acid. Its elementary composition and the barium content of its barium salt are in fair agreement with those required for an oestriolglucuronic acid ($C_{24}H_{32}O_9$). Since it does not reduce Benedict's solution (but does so after hydrolysis) it is clear that the oestriol is linked to the glucuronic acid by a glucosidic linkage through the aldehyde group of the latter. Since the compound gives a strong Millon test in the cold, this linkage possibly does not involve the phenolic hydroxyl of the oestriol unit.

¹ The authors are grateful to Dr Helen Stantial for carrying out this determination.

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XIII. DEPOSITION OF STRONTIUM SALTS IN HYPERTROPHIC CARTILAGE *IN VITRO*.

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EXPERIMENTS on the deposition of barium, strontium and magnesium salts in bone slices *in vitro* have been previously reported [Robison and Rosenheim, 1934] and have shown that the calcifying mechanism is not entirely specific for the calcium salt of bone, although the properties of this salt are very specially appropriate to the function which it fulfils. Deposits of the phosphates (or carbonatophosphates) of these allied metals were produced in the matrix of the hypertrophic cartilage by agency of the phosphatase mechanism. The behaviour of strontium showed further resemblance to that of calcium in that its salts could also be deposited in the cartilage from supersaturated inorganic solutions, in absence of added phosphoric ester, that is, by the agency of the second mechanism.

This reaction of hypertrophic cartilage towards strontium salts gained interest as a result of the experiments of Sobel *et al.* [1934] who showed that the substitution of SrCO_3 for CaCO_3 in a rickets-producing diet caused the development in rats of a severe type of rickets which was not cured by vitamin D therapy. Bone slices from these rats also failed to become calcified *in vitro* in artificial sera which produced good deposits in bone slices from rats suffering from rickets of the usual type. It was subsequently shown [Sobel *et al.*, 1935, 1, 2] that there is a marked diminution, but not a complete destruction, of the calcifying power of the bones of animals suffering from strontium rickets and that the activity of the calcifying mechanism can be restored both *in vivo* and *in vitro* by bathing the bone cells in fluids free from strontium. It was shown that Sr^{++} strongly inhibits calcification *in vitro* but does not affect the activity of phosphatase even in concentrations as high as 80 mg. Sr^{++} /100 ml. These authors suggest that Sr combines with a factor in the bone whose concentration plays a part in calcification.

In view of these results it appears worth while to describe briefly some further experiments which confirm and extend our previous findings although they do not provide the full information for which we sought, namely the exact composition of the deposits. We first investigated the possibility that the deposits obtained in the earlier experiments and presumed to consist of strontium salts were in reality calcium salts, the calcium being derived either from the strontium chloride used in preparing the solutions or from the calcified portions of the bone slices themselves. Specimens of the strontium chloride previously used were submitted to spectrographic analysis and were found to contain appreciable amounts of calcium; the deposits produced in hypertrophic cartilage by solutions prepared from this salt were however shown to contain strontium. It is a reasonable assumption that even if the values of the product $\text{Ca} \times \text{P}$ in these solutions were high enough for calcification *in vitro* in absence of Sr (and this is very unlikely), calcification would have been completely inhibited by the high concentration of Sr.

Other specimens of strontium chloride, supplied by well-known firms, were also found to contain calcium; but a satisfactory specimen ("Specpure") was

obtained from Messrs A. Hilger, Ltd. The degree of purity of this salt was 99.993%; it contained only 0.0013% Ca reckoned on its Sr content. With this salt our further experiments were carried out.

The technique was similar to that previously described. Slices from the heads of the tibiae and humeri of rachitic rats were immersed for periods of 10–22 hours in the solutions at 37° and p_H 7.2–7.3; this p_H was slightly lower than usual in order to lessen the marked tendency of the strontium solutions to spontaneous precipitation. With 0.01 *M* or 0.005 *M* Sr, dense deposits extending through most of the zone of hypertrophic cartilage were uniformly obtained with solutions containing glycerophosphoric ester equivalent to 30 mg. P/100 ml. but no inorganic phosphate. Deposits were also obtained with regularity in absence of ester with solutions containing Sr and inorganic phosphate in suitable concentrations. The $Sr \times P$ product (500–900) was much higher than the $Ca \times P$ product required for calcification *in vitro*. For deposition in absence of ester a basal solution containing 0.6% $NaHCO_3$ and 0.6% or 0.4% $NaCl$ was again found to give the best results. Basal solution B, similar to that used for calcification *in vitro* [Robison and Rosenheim, 1934, p. 685] and containing 0.22% $NaHCO_3$ but no sulphate, gave rather poor deposits with general precipitation. Typical results of these experiments are shown in Table I.

Table I. *Deposition of Sr salts in hypertrophic cartilage in vitro.*

Concentration of $SrCl_2$ ("Specpure")	g. per 100 ml. $NaHCO_3$	mg. per 100 ml.		Deposition in cartilage (max. 10 \times)	$Sr \times P$
		P inorganic	P organic		
0.01 <i>M</i>	0.6	7	0	8 \pm , 9 \pm	616
0.01 <i>M</i>	"	8	0	9 \pm , 6 \pm	704
0.005 <i>M</i>	"	12	0	6 \pm , 5 \pm	524
0.005 <i>M</i>	"	16	0	7 \pm , 7 \pm	706
0.0025 <i>M</i>	"	30	0	8 \pm	660
0.0025 <i>M</i>	"	40	0	7 \pm	880
0.01 <i>M</i>	0.22 (B)	9	0	1 \pm	792
0.01 <i>M</i>	"	10	0	5 \pm	880
0.005 <i>M</i>	"	16	0	2 \pm	706
0.0025 <i>M</i>	"	30	0	2 \pm	660
0.0025 <i>M</i>	"	40	0	3 \pm	880
0.01 <i>M</i>	0.05	0	30	9 \pm , 9 \pm , 10 \pm	
0.05 <i>M</i>	"	0	30	5 \pm	

Deposition in hypertrophic cartilage free from calcified tissue.

In order to eliminate as far as possible the objection that calcium might be derived from the bone slice itself, experiments were also carried out with segments of cartilage dissected from the metaphyses of tibiae and free from all calcified tissue. Deposition occurred in these segments as regularly and as heavily as in the usual bone slices. Spectrographic analysis showed that these cartilage segments, when removed from the bone, contained only traces of calcium, in amounts of the order 0.001–0.003 mg.

Nature of the deposits formed in hypertrophic cartilage in vitro with the experimental strontium solutions.

The spectrographic examination of the deposits obtained in the earlier experiments was carried out by the method of Ramage [1929; Sheldon and Ramage, 1931]. In the later experiments a method of spark spectrum analysis was employed, using copper electrodes. The zone of hypertrophic cartilage containing the deposit was dissected under a binocular microscope and placed in 0.5 ml. *N HCl*; after 16–20 hours 0.5 ml. H_2O was added. Inorganic phosphate

was estimated colorimetrically in 0.5 ml. of this extract. Tests carried out by ashing the residual cartilage showed that extraction was complete. For the spectrographic examination, 0.01 ml. of the extract was pipetted on to the electrode and evaporated by gentle warming; three successive exposures, each of 15 sec., were made. The standard solutions contained Sr, Ca, inorganic phosphate and HCl in concentrations as close as possible to those present in the cartilage extracts. The presence of phosphate had a very marked effect in reducing the intensity of the Ca and Sr lines.

Table II shows some typical results obtained with segments of hypertrophic cartilage from which all calcified cartilage and bone had been dissected away. Three of these segments were immersed for 17 hours in a solution containing

Table II. *Composition of deposits formed in hypertrophic cartilage.*

Composition of solution			Composition of deposits (HCl extracts of cartilage)		
Sr (M)	Inorganic phosphate mg. P/100 ml.	Deposition in cartilage	Sr (mg.)	Ca (mg.)	P (mg.)
0.0025	10	-	0	0.003	0.001
"	"	-	0	"	"
"	"	-	0	"	"
"	40	+	0.26	"	0.040
"	"	+	0.20	"	0.022
"	"	+	0.24	"	0.030

0.0025 M Sr, 40 mg. P/100 ml. as inorganic phosphate, 0.6 g. NaHCO_3 and 0.6 g. NaCl per 100 ml. The other three segments, which served as controls, were immersed for a similar period in a solution containing too low a concentration of phosphate (10 mg. P/100 ml.) to yield any deposit in the cartilage. Great accuracy cannot be claimed for the values for Sr so far obtained by this method, but they undoubtedly prove that the deposits formed in cartilage consist largely of strontium phosphate or carbonatophosphate. The values of the ratio Sr : P suggest that the proportion of carbonate may be higher in these deposits than in the bone salt, but a definite opinion on this point cannot be expressed until more reliance can be placed on the results of the spectrographic analyses. It would seem that the cartilage itself firmly retains a trace of calcium which is not removed by immersion in the strontium solutions.

SUMMARY.

1. The deposition of strontium salts in hypertrophic cartilage *in vitro*, by immersion of the cartilage in solutions containing strontium, inorganic phosphate and carbonate ions, has been confirmed, using spectroscopically pure strontium salts and cartilage dissected away from all calcified tissue.

2. The deposits so formed have been shown by spectrum analysis to consist largely of a strontium phosphate or carbonatophosphate.

We are very greatly indebted to Mr H. Ramage and to Dr N. Lucas for their advice and generous help in carrying out the numerous spectrographic analyses.

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XIV. THE INFLUENCE OF CHANGES INDUCED BY CHOLESTEROL UPON THE CALCIFICATION *IN VITRO* OF RABBIT AORTA.

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It was previously shown [Rosenheim and Robison, 1934] that the aortae of normal rats and rabbits can be calcified *in vitro* by immersion in solutions of similar composition to those used for calcification of hypertrophic cartilage. The course of the process is not the same in these different tissues. In cartilage and osteoid tissue, which normally become calcified *in vivo*, calcification *in vitro*, in suitable solutions, begins rapidly and may be complete in 12 to 24 hours; after such periods of immersion a marked diminution in the power of the calcifying mechanism becomes evident [Robison and Rosenheim, 1934]. Calcification of the aorta *in vivo* is an abnormal phenomenon, and *in vitro* the process is delayed and erratic. Deposition rarely begins until the tissue has been immersed for two days and may not occur within 4 or 5 days, even in solutions which are much more highly supersaturated with calcium carbonatophosphate than those required for deposition of this salt in hypertrophic cartilage. From a consideration of these and other facts it was suggested that conditions or properties favourable to calcification may be developed in the aorta as the result of injury or of degenerative changes. Some attempts were made to obtain experimental evidence in support of this view by subjecting the aorta to various treatments after removal from the animal; these attempts were unsuccessful.

It was decided to continue these experiments by producing in the living animal changes in the aorta which are commonly associated with eventual calcification. The choice of cholesterol sclerosis for our first experiments was taken with the helpful advice of Prof. J. B. Duguid to whom we express our thanks. Experimental cholesterol arteriosclerosis in rabbits simulates in many respects human atherosclerosis [Anitschkow, 1933]. It is characterised by deposition of lipid substances, probably consisting of cholesterol and cholesteryl esters, in the intima, which becomes greatly thickened with formation of large, fat-laden "foam" cells. Fatty plaques are formed on the inner surface, beginning in the arch and extending into the thoracic aorta and abdominal aorta, ultimately covering large areas of the vessel wall. At later stages, calcification of the degenerate tissues often occurs. The earliest microscopical changes may be observed in aortae of rabbits which have received cholesterol over periods of 20 days and upwards. This type of arteriosclerosis is associated with disturbance of cholesterol metabolism, but some form of injury or alteration of the aortic tissues is commonly supposed to play a part in predisposing these tissues to the infiltration of lipoids.

The problem which we set out to study was whether such changes as are induced in the rabbit aorta by diets rich in cholesterol provide conditions more favourable for the deposition of calcium salts than are found in the normal tissues. The plan of our experiments was to compare the rapidity, extent and

character of the calcification *in vitro* produced by supersaturated solutions of the bone salt in aortae of normal rabbits and of those suffering from experimental cholesterol arteriosclerosis in varying degrees of severity.

EXPERIMENTAL

Twelve rabbits, each about 1 year old, were divided into pairs of approximately the same weight and were given a diet consisting of oats, bran, hay, cabbage and carrot. One of each pair received in addition 1 g. cholesterol daily, this being thoroughly mixed with minced carrot and eaten by the animals before they received the rest of the food. Five pairs of rabbits were killed after periods of 35, 43, 50, 81 and 91 days respectively. One rabbit of the sixth pair received cholesterol during 112 days; thereafter it received the normal diet without cholesterol for a further 41 days and was then killed along with the rabbit which had received the normal diet for the whole period. Immediately after death the aortae were excised with aseptic precautions and were divided transversely into segments some of which were placed in the calcifying solution, whilst the remainder were fixed at once as controls. The whole of the ascending aorta and arch, the thoracic aorta and a small part of the abdominal aorta were used, the segments being numbered consecutively, beginning from the heart.

The calcifying solution (8 : 5 : 0) used in all experiments contained 8 mg. Ca and 5 mg. P, as inorganic phosphate, per 100 ml., together with other inorganic salts in the concentrations in which they occur in plasma [Robison and Rosenheim, 1934]. Each segment was placed in a tube containing 18 ml. of this solution at p_H 7.4 and 37°. In the previous investigation [Rosenheim and Robison, 1934] solution 8 : 5 : 0 produced calcified deposits in 5 days in 3 out of 6 rabbit aortae.

In the first three experiments of the present series each aorta was divided into four segments: the first and third were immersed in solution 8 : 5 : 0 for 6 days, whilst the second and fourth served as controls. In Exps. 4-6 the aortae were divided into ten smaller segments and the period of immersion was varied from 1 to 5 days in order that any increased tendency to calcification might be more readily detected. In order to minimise the risk of spontaneous precipitation of calcium salts the experimental solution was renewed at intervals of 1 or 2 days.

The segments were fixed in 10% neutral formalin, dehydrated and embedded in paraffin. Serial sections were cut and stained with silver nitrate and eosin; alternatively, some sections were stained with purpurin and orange G or with haemalum and eosin. In Exps. 4-6 some control segments were cut with the freezing microtome and stained with Sudan III and haematoxylin for the detection of lipoids.

The results of these experiments are summarised in Table I.

Changes induced by cholesterol.

Extensive fatty plaques were found in all segments of the aortae of rabbits which had received cholesterol for periods of 81, 91 and 112 days (Exps. 4-6). Considerable deposits of lipoids were present in the intima and in the inner layers of the media. Definite, though less extensive, intimal lesions were observed in the aortae of the rabbits which had received cholesterol for 35, 43 and 50 days (Exps. 1-3). The calcification which often accompanies the later stages of cholesterol arteriosclerosis did not appear in any of these experiments; no calcified deposits were seen in any segments which had not been immersed in calcifying solution.

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Table I. *Calcification in vitro of rabbit aorta.*

		Segments immersed in solution 8 : 5 : 0										
Exp.	Sex	Initial wt. g.	Final wt. g.	Diet	Period on diet days	Control segments			Nos. of seg- ments	Period of immer- sion days	Calcification	
						Nos. of segments	Calcifi- cation	Chol- esterol lesions				
1	♂	2530	2890	N	35	2, 4	-	-	1, 3	6	++	i, m, a
		2550	2730	C	35	2, 4	-	+	1, 3	6	++	i, m, a
2	♂	3470	3050	N	43	2, 4	-	-	1, 3	6	++	i, m, a
		3620	3020	C	43	2, 4	-	+	1, 3	6	++	i, m, a
3	♀	2050	2520	N	50	2, 4	-	-	1, 3	6	++	i, m, a
		2525	3200	C	50	2, 4	-	+	1, 3	6	++	i, m, a
4	♂	3080	2835	N	81	3, 6, 9 2, 5, 8	-	-	1	1	-	
									4	2	-	
									7	4	-	
									10	5	-	
	♀	3300	2850	C	81	3, 6, 9 2, 5, 8	-	+	1	1	-	
									4	2	-	
									7	4	-	
									10	5	++	i, m, a, p
5	♂	2845	2990	N	91	3, 6, 9 2, 5, 8	s	-	1	1	s	m
									4	2	-	
									7	3	-	
									10	4	+	m
	♀	2970	2575	C	91	3, 6, 9 2, 5, 8	-	+	1	1	s	m
									4	2	+	p
											(Few granules)	
									7	3	-	
									10	4	+	m
6	♂	2795	3570	N	153	3, 6, 9 2, 5, 8	-	-	1	1	-	
									4	2	++	m
									7	3	++	m
									10	5	-	
	♀	2685	2820	C N	112 41	3, 6, 9 2, 5, 8	-	+	1	1	s	m
									4	2	-	
									7	3	++	m
									10	5	++	i, m, a

Diet: N, normal; C, normal + 1 g. cholesterol daily.

Calcification: -, no deposit; +, very light deposit; ++, heavy deposit; s, very small calcified area occurring *in vivo*.

The letters indicate the location of the deposit: i, intima; m, media; a, adventitia; p, fatty plaque.

The most extensive deposition was almost always found in the media.

In Exps. 4-6, segments 3, 6, 9 were cut with the freezing microtome and examined for lipid deposits.

In the aorta of the normal rabbit of Exp. 5, a very small area of calcification, involving one or two elastic laminae in the media, was found in segment 2 (arch) which had been fixed as a control (Plate I, Fig. 1). Very similar calcified areas were seen also in segment 1 of the same aorta and in the first segments of the aortae of two rabbits receiving cholesterol (Exps. 5 and 6). Although the last three segments had been immersed in solution 8:5:0 for one day, it was considered that the deposits were most probably formed *in vivo* but were not necessarily induced by the cholesterol diet. This type of calcification is indicated in Table I by the letter "s". The presence of such deposits in the aortae of apparently normal rabbits has been frequently noted. In a recent study, Kesten [1935] observed spontaneous medial degeneration in the aortae of 68 out of 125 apparently normal young rabbits aged from 1 day to 8 months. Histologically, the

earliest changes were swelling, fragmentation and fraying of elastic fibres in the inner media, and at later stages calcium salts were laid down along and closely apposed to these swollen fibres.

Calcification in vitro.

In Exps. 1-3, in which alternate segments were immersed for 6 days in solution 8 : 5 : 0, heavy calcification was obtained *in vitro* in the aortae of the normal rabbits and of those which had received cholesterol. As in the earlier experiments [Rosenheim and Robison, 1934] the deposits were found chiefly in the media but occurred also in the intima and adventitia, sometimes extending throughout the whole width of the wall. In lightly calcified areas the appearance of the stained deposit was that of scattered granules formed on or around the elastic fibres. This was shown particularly well in sections stained with purpurin in which fibres could be seen through the translucent pink granules (Plate I, Fig. 2). In the more densely calcified areas the deposit extended between the elastic laminae and frequently appeared to lie chiefly in the muscle tissue. Under high magnification deposits of the latter type showed particles attached to fine offshoots joining the elastic laminae, but whether deposition always began in this way could not be determined. The impression that elastic fibres play an important part in the initiation of calcification in the aorta was strengthened by the examination of these deposits.

In these three experiments the aortae of the normal rabbits and of those which had received cholesterol did not show any significant differences in their *in vitro* calcification.

In Exps. 4-6, in which different segments were immersed in calcifying solution for periods of 1-5 days, calcification *in vitro* occurred erratically. In Exp. 4 no calcification occurred in any segment of the normal rabbit aorta, whilst in that of the rabbit receiving cholesterol a heavy deposit was formed in one segment only, immersed for 5 days, in this segment, calcification took place in some parts throughout the vessel wall and in the extensive fatty plaque (Plate I, Fig. 3). The presence of numerous bacteria was, however, observed in the peripheral layers of this segment and these organisms may conceivably have played some part in determining calcification in these regions; no bacteria were seen in the middle zone where a heavy deposit was also found.

In Exp. 5 a light deposit was observed in the media of one segment of the normal aorta, immersed for 4 days; the corresponding segment of the aorta from the rabbit receiving cholesterol also showed slight calcification after 4 days' immersion, the deposit being formed on the innermost elastic laminae. In another segment of the same aorta, immersed for 2 days only, a few granules of calcium salt were found in the fatty plaque (Plate I, Fig. 4); but no calcification occurred in segment 7 after 3 days' immersion. In Exp. 6 segments of the normal aorta were calcified after 2 and 3 days' immersion (Plate I, Fig. 5) but not after 1 or 5 days, whilst the aorta of the rabbit which had received cholesterol became calcified to about the same extent after 3 and 5 days' immersion but not after 2 days. A section of segment 7, which was immersed for 3 days, is shown in Plate I, Fig. 6. The fat has been removed during dehydration but one end of an extensive intimal plaque is seen. There is no calcification in this plaque or in the sub-intimal layers; but a heavy deposit is present in the middle region of the media and resembles that found in segment 7 of the normal aorta after immersion for a similar period (Plate I, Fig. 5).

Most of the deposits observed in Exps. 4-6 occurred in the media, and granules formed on or around elastic fibres were very conspicuous.

The staining of the deposits.

The staining properties of the deposits of calcium salts (probably calcium carbonatophosphate) formed in these experiments were investigated in some detail. With haemalum the staining was very slight, only the heaviest deposits retaining the colour around their edges; a deeper, though rather patchy, stain was obtained if the sections were previously immersed for some time in a 1% solution of ferric chloride. Purpurin and silver nitrate stained the deposits in approximately the same degree, the purpurin giving a deep clear pink colour, characteristic of calcium in absence of iron. The greatest reliance was placed on silver nitrate but the method has certain defects. With heavy deposits, there is a tendency to spreading of the brown stain into surrounding tissues; whilst, occasionally, the reduced silver may form relatively large and apparently crystalline particles, which probably do not show the exact distribution of the original calcium salt. As pointed out by Cameron [1930] it is not the calcium ion but the anion, carbonatophosphate, which reacts with silver nitrate.

Through the kindness of Dr C. V. Harrison, we were able to compare these reactions with those given under precisely similar conditions by the deposits formed *in vivo* in his experiments on arterial disease produced by cholesterol and vitamin D [Harrison, 1933]. In sections cut from his material, all deposits stained by silver nitrate were also stained by haemalum and purpurin, the latter giving a reddish purple colour indicating the presence of iron. In addition, certain areas not stained by silver nitrate were stained by haemalum and showed also a faint purple stain with purpurin. Cameron [1930] concluded that "haematoxylin does not stain calcium salts though it often identifies areas in which changes favourable to the deposition of calcium salts are taking place. The reaction obtained in these areas depends partly on the presence of iron and mostly on a peculiar ground substance which is normally present in bone and cartilage and which also appears at the site of pathological calcification and, if mordanted with aluminium or chromium, stains deeply with haematoxylin." In accordance with this view we may consider either that this peculiar ground substance was not present in the aortae used for our experiments and did not develop *in vitro* as a preliminary to calcification, or that the absence of iron from our experimental solution was the sole cause of the failure to stain with haemalum. These alternatives will be investigated in further experiments.

The possible influence of bacteria in the tissues upon calcification.

The precautions taken to avoid bacterial contamination of the excised aortae were not so successful as in similar experiments with bone slices. Many of the calcifying solutions when tested at the end of the experimental period produced bacterial growths on agar slopes at 37°. Since the solutions were sterilised before use, the bacteria must have been introduced with the tissue segments. The contamination was not sufficient to produce visible turbidity in the solutions or to affect their p_{H} .

Microscopical examination of these segments revealed the presence of bacteria in the surface layers of intima and adventitia; occasionally the contamination extended for some little distance into the media. Rarely could any association be traced between the areas so contaminated and those in which calcium deposition took place. The chief instance of such possible relationship was seen in Exp. 4, segment 10 (Plate I, Fig. 3); but even here, dense calcification also occurred in the middle region where no bacteria were seen.

The effect of bacterial invasion on the calcification of tissues *in vitro* is a question of sufficient interest to warrant further study. It is, however, reasonably certain that the formation of most of the deposits observed in our experiments was not due to this cause.

DISCUSSION.

From the results of these experiments it appears that the specific changes induced in the rabbit aorta *in vivo* by diets rich in cholesterol do not provide conditions more favourable for the deposition of calcium carbonatophosphate *in vitro* than are present, or may develop *in vitro*, in aortae excised from normal rabbits.

Neither the lipid deposits characteristic of cholesterol arteriosclerosis nor the tissues in which these lipoids occur have proved specially prone to calcification in our experimental solutions, although deposits of calcium salts were occasionally produced in these areas. This conclusion does not stand in contradiction to the fact that calcification often occurs *in vivo* in atheromatous lesions of long standing which have undergone further necrotic changes. Our experiments were probably of too short duration for such secondary degenerative changes to take place.

It is believed [Duff, 1935] that some alteration of the tissues precedes and favours the deposition of lipoids in this type of arteriosclerosis. We consider that some alteration of the tissues may also precede and favour deposition of calcium salts in the aorta *in vivo* and *in vitro*: but our present results do not justify the assumption that changes which lead to or accompany the accumulation of lipoids in the aorta have any immediate relationship to its subsequent calcification.

The histological interpretation of calcification produced *in vitro* is made difficult by the cell degeneration which accompanies the prolonged immersion of the tissues in the experimental solutions; after 5 or 6 days few stainable nuclei remain. It is permissible, however, again to emphasise that the phenomenon has certain aspects in common with calcification occurring in vitamin D sclerosis as described by Duguid [1930] and others. Both processes are associated with high values of the product $\text{Ca} \times \text{P}$ in the fluids bathing the tissues: in both, calcification affects, primarily, the media, picking out first the elastic laminae and later extending into the tissue between them. Harrison induced cholesterol sclerosis and vitamin D sclerosis successively in the same rabbit and found that the respective lesions were confined to different parts of the vessel. In some of his experiments the two lesions, intimal and medial, alternated in an almost unbroken chain around the circumference of the aorta, a result attributed by him to the movements of the vessel and the relative immobility of those areas affected by the preliminary lesion, rendering them less susceptible to the subsequent lesion. Factors of this nature were not operative in our experiments. In Harrison's experiments, calcification was sometimes found in the fatty debris in the deeper parts of the cholesterol plaques and was more marked in those rabbits which had subsequently received irradiated ergosterol. With these observations, apart from the differences inherent in the two types of experiments, our own results are in agreement.

From information kindly given to us by Prof. I. M. Heilbron it appears that the "pure cholesterol" of commerce is not entirely homogeneous but contains about 0.1% of another sterol which shows selective absorption similar to ergosterol but is probably not identical with the latter. The quantity of cholesterol which must be administered to rabbits in order to produce athero-

matous lesions in the aorta is so large in comparison with the quantity of calciferol which produces medial calcification that the possibility that the second sterol, although present in such small proportion, may play some significant part in the development of experimental cholesterol sclerosis would seem to be worth investigation.

SUMMARY.

1. The influence of the changes induced in the rabbit aorta *in vivo* by diets rich in cholesterol on subsequent calcification has been investigated by the method of calcification *in vitro* previously described.

2. The development of typical cholesterol arteriosclerosis was not found to be associated with any increased proneness to calcification of the aorta *in vitro* nor did the deposits so obtained specially favour those portions of the vessel in which the cholesterol lesions occurred.

We wish to thank Dr C. V. Harrison and Dr E. Weston Hurst to whom we are indebted for much helpful advice on the histology of this investigation. Our thanks are also due to Messrs Glaxo Laboratories Ltd., who kindly provided the cholesterol used in these experiments.

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DESCRIPTION OF FIGURES IN PLATE I.

ca, calcification; e.l., elastic lamina; p, cholesterol plaque.

- Fig. 1. Exp. 5. Ascending aorta of normal rabbit, segment 1, showing calcification in the media on elastic laminae (occurring *in vivo*). AgNO₃ and eosin. $\times 280$.
 Fig. 2. Exp. 2. Ascending aorta of rabbit which had received 1 g. cholesterol daily for 43 days; segment 1, 6 days in solution 8 : 5 : 0. Note typical appearance of granules of Ca salt deposited on the elastic laminae in the media. Purpurin and orange G. $\times 280$.
 Fig. 3. Exp. 4. Abdominal aorta of rabbit which had received 1 g. cholesterol daily for 81 days; segment 10, 5 days in solution 8 : 5 : 0. Note extensive calcification in intima, media, adventitia and in the large intimal plaque. Numerous bacteria were present in the surface layers of the segment. AgNO₃ and eosin. $\times 30$.
 Fig. 4. Exp. 5. Intimal plaque, in side artery at junction with aortic arch, of rabbit which had received 1 g. cholesterol daily for 91 days; segment 4, 2 days in solution 8 : 5 : 0. Note granules of calcium salt deposited in plaque. AgNO₃ and eosin. $\times 520$.
 Fig. 5. Exp. 6. Thoracic aorta of normal rabbit, segment 7, 3 days in solution 8 : : 0. Note calcification in the media, on and between the elastic laminae. AgNO₃ and eosin. $\times 120$.
 Fig. 6. Exp. 6. Thoracic aorta of rabbit which had received 1 g. cholesterol daily for 112 days, then normal diet for 41 days; segment 7, 3 days in solution 8 : 5 : 0. Note calcification in the media, but none in the intima or plaque. AgNO₃ and eosin. $\times 120$.

XV. THE KINETICS OF ALCOHOLIC FERMENTATION OF SUGARS BY BREWER'S YEAST.

IV. SPECIFICITY. THE RATES OF FERMENTATION OF α - AND β -GLUCOSE.

BY REGINALD HAYDN HOPKINS
AND RICHARD HENRY ROBERTS.

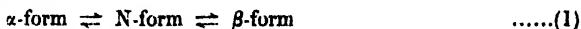
From the Department of Industrial Fermentation, University of Birmingham.

(Received November 30th, 1935.)

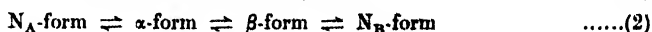
ARMSTRONG [1904] advanced the hypothesis that the specificity exhibited by yeast towards glucose, mannose and fructose was due to the existence of an enol form common to the three hexoses. Hopkins [1931] developed a modification of this hypothesis to show certain features of configuration common to α -glucopyranose, α -mannopyranose and fructofuranose using the configurations of Haworth. In the case of glucose, Willstätter and Sobotka [1922] and Hopkins [1931] had shown that, when a solution of glucose is fermented rapidly by brewer's yeast and the fermentation is interrupted, the residual solution exhibits mutarotation in the positive sense. This was interpreted to mean that the yeast selectively preferred the α -form of glucose. Hopkins [1931] had further shown that fructose, partly fermented by brewer's yeast, also exhibited mutarotation in the positive sense. The inference was drawn that brewer's yeast specifically ferments a form of fructose possessing a less negative specific rotation than that of fructose at equilibrium: this was quite possibly fructofuranose which shows a certain configurational relationship with glucopyranose and mannopyranose.

The simplest explanation of these phenomena is to suppose that brewer's yeast specifically ferments α -glucose, α -mannose (for which the evidence will be furnished in a later communication) and some form of fructose. However, there is the alternative explanation that, whilst both α - and β -forms of the sugars are directly fermentable, the α -form is preferred just as glucose is preferred to fructose in mixtures of the two. Certain experiments of Willstätter and Sobotka [1922] seem to support this, in that α - and β -glucose separately fermented at the same rate, just as glucose and fructose do. However, these fermentations were conducted at concentrations high enough to prevent concentration of sugar from being the limiting factor. Lastly there remains the possibility that intermediate forms of the sugars are specifically attacked.

In recent years evidence has been forthcoming to strengthen this last hypothesis. Lippich [1932] measured the HCN-binding powers ("Blausäurezahl") of sugar solutions and concluded that open-chain forms were present in appreciable amounts. One or, more probably, two such "N-forms" may exist. Thus for glucose he suggests either

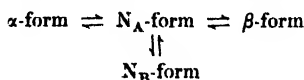


or



the N_A and N_B forms being presumably open-chain forms related stereochemically in some such manner as are α - and β -glucose. Although Lippich was forced to

consider (2) to be more representative of a glucose solution, it is simpler for our purposes to make use of (1), or of a modification of (2) in the form,



the N_A form being that specifically fermented by brewer's yeast.

With a view to deciding between the three explanations outlined above of the specificity of brewer's yeast for glucose it was considered desirable to measure the rates of fermentation of α -, β - and $\alpha\beta$ -glucose, all at concentrations low enough to influence or even to control the rate of fermentation.

EXPERIMENTAL.

The apparatus used for measuring velocities of fermentation was that used by Slator [1906] with modifications and technique as described by Hopkins and Roberts [1935, 1]. In the fermentation vessel the brewer's yeast in suspension was brought to the desired temperature and the sugar, in solid form or in solution at equilibrium, was contained in a small beaker on the float on the surface. This sugar was discharged into the yeast suspension by starting the shaker after exhaustion of the system. α -Glucose was recrystallised from alcohol, dried *in vacuo* over P_2O_5 and finally desiccated in a Fischer drier. β -Glucose was crystallised from pyridine, dried and freed from pyridine at 100° for 2 hours, its identity being confirmed by polarimetric observations.

The rates of fermentation were observed by reading the manometer at intervals of 0.5–2 min. and calculated to mg. CO_2 /min. using the appropriate calibration factor for the total volume and temperature of liquid employed [Hopkins and Roberts, 1935, 1].

The results in Tables I and II show that α -, β - and $\alpha + \beta$ -glucose at 1 % concentration are fermented at 30° at the same rate. However, in the case of $\alpha + \beta$ -glucose in equal proportions, rather less time was required to attain

Table I. *Rates of fermentation of α - and β -glucose.*

1 g. α - or β -glucose; 4 g. yeast; 100 ml.; 30° ; CO_2 as mg. min.

Time (min.) ...	2	5	10	15	20	25	30	35
α -glucose	3.40	4.98	5.29	5.23	5.18	5.12	4.93	4.7
β -glucose	3.40	4.95	5.32	5.23	5.20	5.15	4.93	4.75

Table II. *Rates of fermentation of (1) α -glucose, (2) an equimolar mixture of α - and β -glucose.*

(1) 0.5 g. α -glucose, (2) 0.25 g. α -glucose + 0.25 g. β -glucose. 4 g. yeast; 50 ml.; 30° ; CO_2 as mg. min.

Time (min.) ...	1	2	4	6	8	10	15	20
α -Glucose	0.26	1.04	2.08	2.81	3.30	3.74	4.47	4.94
$\alpha + \beta$ -Glucose	0.52	1.30	2.29	3.02	3.53	3.95	4.08	5.10
Time (min.) ...	25	30	35	40	45	50	55	60
α -Glucose	5.20	4.99	4.58	4.06	3.54	2.91	1.56	0.41
$\alpha + \beta$ -Glucose	5.20	4.89	4.37	3.95	3.54	2.81	1.56	0.52

the maximum rate than with α -glucose alone. Subsequent fermentations were carried out at 35° , since it was found that with the very high yeast rate that was necessarily employed the induction period was greatly reduced at this temperature. The results of these later experiments are shown in Figs. 1 and 2.

DISCUSSION.

Selective fermentation of $\alpha\beta$ -glucose, as observed by the mutarotation of the partly fermented sugar, indicates that the α -form is removed more rapidly than the β -form [Willstätter and Sobotka, 1922; Hopkins, 1931]. The results however do not support the simple hypothesis that brewer's yeast is specific for α -glucose. For example, calculation shows that in the case of β -glucose at 35° and p_H 5.0–6.0 (Fig. 1) the 1% solution would after 6 min. unmolested mutarotation have yielded about 0.11% of α -glucose. At the same stage the α -glucose (Fig. 1) would have produced about 0.22% of β -glucose, leaving 0.78% of α -glucose unchanged. If the yeast were specific for the α -form, the rate of fermentation of the former sugar solution at this stage would be the same as that of $\alpha\beta$ -glucose containing 0.11% of the α -form, i.e. 0.30% $\alpha\beta$ -glucose, and the rate for the latter solution the same as that of 2.1% $\alpha\beta$ -glucose. The latter would show the greater rates of fermentation if the yeast were in any way specific for the α -form. However, after 6 min. the maximum rates of fermentation were attained in each case and were the same. These experiments prove this equality more clearly than the corresponding ones of Willstätter and Sobotka [1922] who employed much higher concentrations of the sugars and measured the times of half-fermentation.

In these earlier experiments variations in yeast rate and temperature at 1% sugar concentration were introduced. The only difference revealed between the sugars investigated (α -, β -, $\alpha + \beta$ - and $\alpha\beta$ -glucose) was between $\alpha\beta$ -glucose on the one hand and freshly dissolved α - or β -glucose on the other at 35° (Fig. 1).

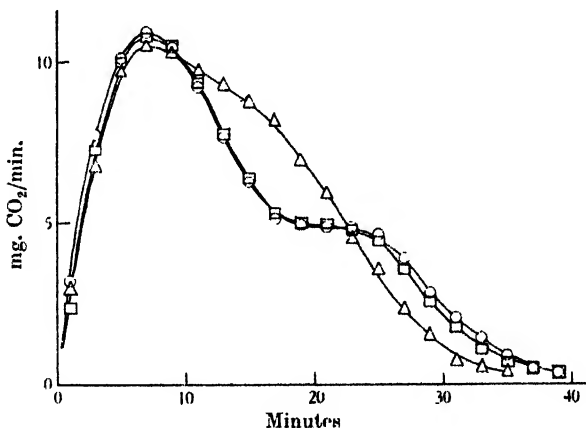


Fig. 1. Rates of fermentation of α -, β - and $\alpha\beta$ -glucose respectively, at 1% concentration. 0.5 g. α -, β -, or $\alpha\beta$ -glucose; 8 g. yeast; 50 ml.; 35° . α - \circ , β - \square , $\alpha\beta$ - \triangle .

These conditions were adopted since, from a consideration of the temperature coefficient of mutarotation and the observed induction periods, it was apparent that the maximum rate of fermentation would then be attained with minimum mutarotation. The distinctive appearance of the curves (Fig. 1) for α - and β -glucose would seem to be due to the relative velocities of fermentation and mutarotation, since under other conditions (Tables I and II) the curves were of the usual compound exponential type and resembled those of $\alpha\beta$ -glucose in all experiments. The times of half-fermentation are the same in all three cases, viz. 12, 11.9 and 12.1 min. for α -, β -, and equilibrium glucose respectively, a result

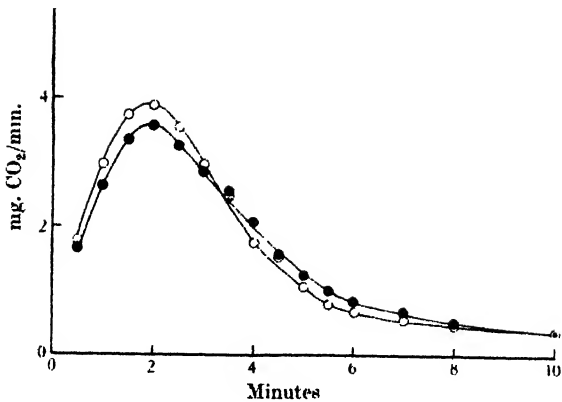


Fig. 2 a.

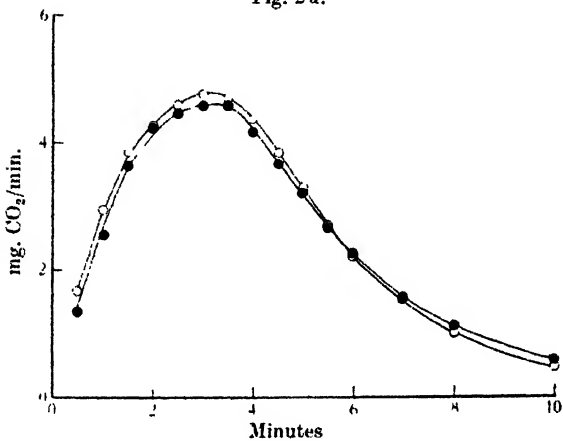


Fig. 2 b.

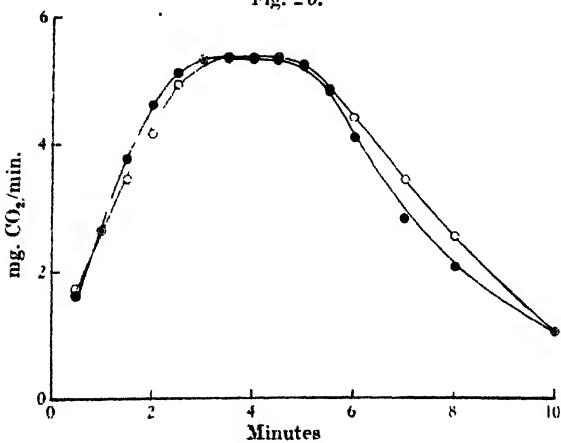


Fig. 2 c.

Fig. 2 (a, b, c). Rates of fermentation of α - and β -glucose at different concentrations.
8 g. yeast: 50 ml.; 35°.

	a	b	c
α -Glucose (o)	0.252 %	0.508 %	0.753 %
β -Glucose (•)	0.255 %	0.501 %	0.749 %

words, the β -form is not contributing its share, so that the proportion of α - and β -forms becomes changed to one with an abnormally high proportion of β -glucose. On arresting the fermentation, mutarotation to equilibrium follows, as observed. Some evidence in support of this explanation is furnished in Fig. 2, which is discussed below. Alternatively, the changes of rotation in partly fermented glucose solutions can be accounted for by postulating merely that the specific rotation of the N-form is higher than that of $\alpha\beta$ -glucose. The removal of this form would have the effect observed. If however the N-form is present in only a relatively low proportion, then its specific rotation must be high. Both of these explanations may apply to the case, the resultant of the two effects being the mutarotation observed.

The above explanation of the phenomenon of selective fermentation of $\alpha\beta$ -glucose assumes, as stated, that 35 parts of the α -form would in a given time produce the same amount of the N-form as would 64 parts of the β -form. If this is so, then, if sufficiently low concentrations are employed, α -glucose should ferment separately faster than β -glucose. A concentration of 1% is not low enough, but Fig. 2 (*a, b, c*) clearly shows this effect, progressively increasing as concentration is lowered from 0.75 to 0.25%. Application of the Michaelis and Menten theory as described by Hopkins and Roberts [1935, 1], and plotting the reciprocals of maximum velocity as ordinates against reciprocals of initial concentration, yielded the graphs in Fig. 3. From these, following the procedure described by Hopkins and Roberts [1935, 2], the following values of K_m , the Michaelis and Menten constant, were calculated:

α -glucose, 0.178 corresponding to 0.0099 *M*.

β -glucose, 0.212 corresponding to 0.0118 *M*.

That the ratio of these, 0.21 : 0.18, is not as great as that of 64 : 35 is not surprising since, firstly, the observed maximum velocities were attained only after a certain amount of mutarotation had taken place, secondly, the ratio 64 : 35 for

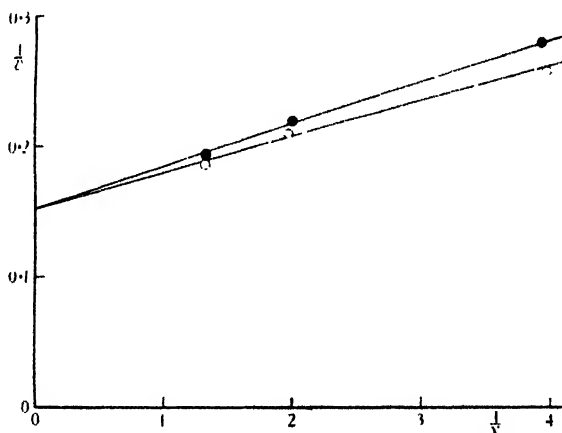


Fig. 3. α -glucose \circ , β -glucose \bullet .

the proportions of β - and α -glucose respectively in solutions at equilibrium may be far from correct, and thirdly, experimental errors are appreciable. Nevertheless, the results of Figs. 2 and 3 support the former of the two explanations furnished to account for the phenomenon of the mutarotation of partly fermented $\alpha\beta$ -glucose.

SUMMARY.

1. The maximum rates of fermentation attained by α -, β - and $\alpha\beta$ -glucose are the same unless the initial concentration of sugar is well below 1 %.
2. With initial concentrations of 0.5 and 0.25 % the fermentation of α -glucose attains a maximum rate greater than that attained in the case of β -glucose.
3. The values of the Michaelis and Menten constant, K_m , for fermentation of α - and β -glucose are 0.0099 and 0.0118 M respectively.
4. These observations, and particularly the progress of the fermentations at 1 % concentration, lead to the hypothesis that neither α - nor β -glucose, but an intermediate, possibly open-chain, form of glucose is specifically fermented. The α -form is converted into this fermentable form more rapidly than the β -form.
5. The mutarotation of partly fermented $\alpha\beta$ -glucose may be explained on this hypothesis.

One of the authors (R. H. R.) is indebted to the Trustees of the Fentham Trust for a grant which enabled him to carry out the work recorded in Parts I-IV of this series of papers.

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XVI. THE ULTRAFILTRATION OF PROTEINS THROUGH GRADED COLLODION MEMBRANES.

II. HAEMOCYANIN (*HELIX*), EDESTIN, AND EGG ALBUMIN.

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(Received November 8th, 1935.)

THE behaviours of protein solutions when filtered through membranes of graded porosities under different conditions of p_H and dispersion medium have been further studied in the case of haemocyanin (*Helix pomatia*), edestin and egg albumin. The particle sizes of these proteins in dilute solution have been determined by Svedberg and his co-workers by the method of ultracentrifugal analysis, the respective values being 24, 8 and 4.34 $m\mu$. The purpose of our present study has been to ascertain the conditions most favourable for the filtration of these proteins in a manner similar to that followed for the serum proteins [Elford and Ferry, 1934], and to establish for each the filtration end-point. A means is then provided of checking further the value of the empirical correction factor adopted by Elford [1933] for deducing the particle size of a suspension from the pore diameter of the limiting membrane by which it is just completely retained.

The experimental ultrafiltration technique has been exactly as described in the earlier paper dealing with the serum proteins [1934]. The analyses of filtrates have been based upon refractometric measurements and controlled precipitation tests with salicylsulphonic acid. The stock protein solutions were always sterilised by filtration through a suitable membrane.

Haemocyanin (Helix pomatia).

Three separate batches of crystalline haemocyanin were used. We are greatly indebted to Dr G. A. Millikan for his valued advice and assistance in preparing the crystalline protein. The procedure followed was essentially that outlined by Svedberg and Chirnoaga [1928].

Influence of medium on filterability. The filtration curves have been established for haemocyanin dispersed in the same concentration at p_H 7.3 in each of the four media: water, 1 % saline, *M*/15 phosphate buffer and Hartley's broth. 0.1 *N* NaOH was used to adjust the p_H when necessary. The curves for membranes of average pore diameters (A.P.D.) 125 $m\mu$ are given in Fig. 1. Broth is clearly the best medium for the filtration of haemocyanin but it is very interesting to find that for small volumes of solution (1–2 ml.) the relative filtrate concentration, u , for water as medium may exceed that for broth. However, considerable mechanical blocking of the membrane occurs in the later stages suggesting that whilst the initial adsorption of haemocyanin may be only slight the system is relatively sensitive to influences favouring aggregation. The initial adsorption appears to be considerable in the presence of 1 % saline and the buffer salts, but the broth medium ensures a high value of u_{\max} and its stabilising influence minimises the degree of blocking of the pores.

Influence of p_H . Over a wide range of p_H , 5 to 8, the values of u_{\max} for 0.5 % solutions haemocyanin in broth medium when filtered at 76 cm. mercury pressure

through membranes of A.P.D. 0.16μ were consistently within 95 to 100%. Below p_H 6.0 the initial adsorption zone increased progressively, but the value of u_{\max} was not affected until p_H values below 5.0 were reached, when an abrupt

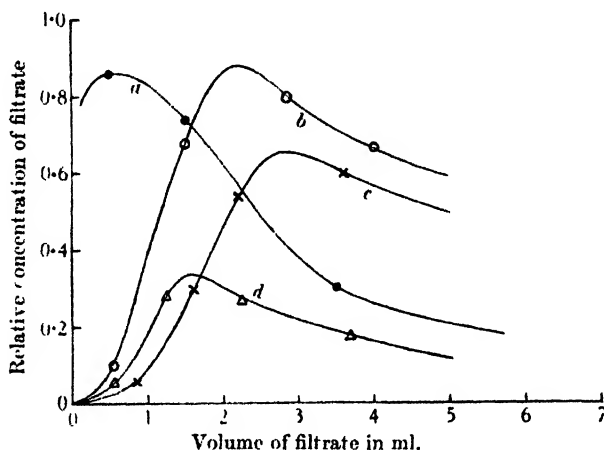


Fig. 1. Filtration curves of 0.75% haemocyanin in different media. (a) water p_H 7.30; (b) broth p_H 7.31; (c) 1% saline p_H 7.28; (d) M/15 phosphate buffer p_H 7.33. Membrane characteristics A.P.D. $125m\mu$; thickness 0.13 mm. Filtration pressure 76 cm. mercury.

falling off was observed. It was evident that for purposes of establishing the filtration end-point curve, the most favourable medium would be broth at a p_H between 7 and 8.

Filtration end-point data. Table I contains a summary of filtration data for membranes of various porosities enabling the end-point for haemocyanin to be deduced.

Table I.

Haemo- cyanin batch	Medium	Concen- tration %	p_H	Pressure atmos.	A.P.D. $m\mu$	u_{\max} $\frac{u}{u_0}$
A	XD broth	1	7.0	3	84	1-5
	"	"	"	3	55	Nil
	Stock broth	1	6.8	2	115	80
	"	1	6.8	2	84	20
	"	"	7.0	2	70	15
B*	"	1	7.3	1	115	75
	"	"	"	"	75	10
	"	"	"	"	64	1
	"	"	"	"	57	? trace < 1
	"	"	"	"	50	Nil
	Phosphate	1	7.3	1	75	Nil
C	Stock broth	0.5	7.31	1	125	85
	"	"	"	"	85	60
	"	"	"	"	74	55
	"	"	"	"	64	10
	"	"	"	"	57	? trace
	"	"	"	"	54	Nil
	"	"	"	"	50	Nil

* This batch showed great reluctance to crystallise; in experiments with the dialysed solution all membranes of $70m\mu$ and less retained the haemocyanin completely and the filtration curves were markedly abnormal. The stock was re-dialysed against distilled water and a white precipitate formed and collected mainly as a surface layer on centrifuging. The blue opalescent solution of haemocyanin was pipetted into another sac and dialysis continued against acetate buffer, p_H 4.8. Crystallisation of haemocyanin occurred. The results in the table are those given by the recrystallised preparation.

Membranes of A.P.D. below $55m\mu$ retain the protein completely even in broth medium under conditions most favourable for filtration, whilst more porous membranes permit the protein to pass. The filtration end-point for haemocyanin (*Helix*) is therefore $55m\mu$, from which we deduce [Elford, 1933] that the protein has a particle size of 18 to $28m\mu$. Svedberg and Chirnoaga [1928] and also Svedberg and Heyroth [1929], by the method of sedimentation analysis, concluded that the protein particles were spherical and $24m\mu$ in diameter. Thus the ratio of the particle diameter (by sedimentation analysis) to the average pore diameter of the limiting membrane for complete retention of the protein is 0.44.

Edestin.

The edestin was prepared from hemp-seed by extraction with neutral salt solution according to the method of Osborne [1909].

Special difficulties were encountered in the filtration studies with edestin solutions. A high concentration of salt was necessary to dissolve the protein at all, and even so the solubility was small. Further, the edestin solutions became turbid on standing and a precipitate settled out, a process which continued for a month or two until about half the protein had become insoluble. Contact with a collodion membrane appeared to accelerate this process. In the presence of broth the solution was relatively stable, and most of our filtration studies were made in this medium.

Influence of p_H . Edestin may be dissolved in acidulated water directly, in the absence of salt, and then adjusted, by adding alkali, nearly to the isoelectric point before precipitation occurs. Experiments made with solutions in dilute HCl at p_H 2.7 showed that the protein filtered very readily under these conditions. The filtration end-point was $15m\mu$, which is slightly lower than that established for solutions in broth at p_H 7.6. It is interesting to note that the reaction p_H 2.7 is outside the range of stability for monodispersion, p_H 5.5 to 9.7, determined by Svedberg and Stamm [1929]. They found edestin to be quite unstable on the acid side of its isoelectric point (p_H 5.5). The filtration results with acid edestin solutions are in accord with this view.

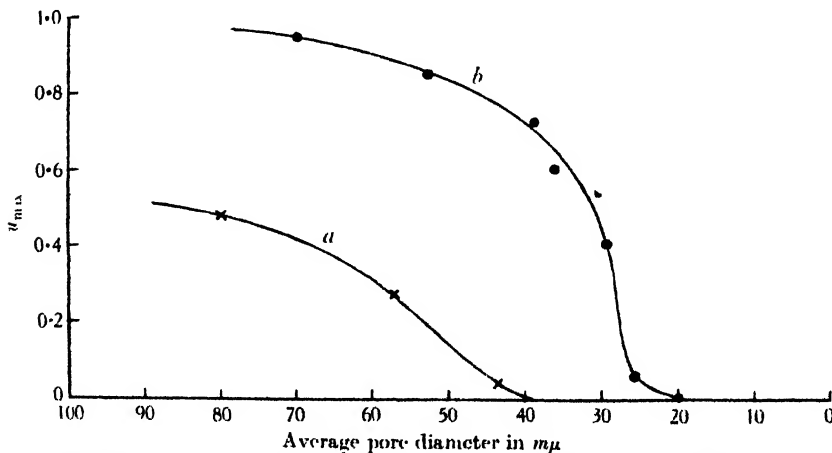


Fig. 2. Filtration end-point curves for edestin. (a) 0.5% edestin in 1.5 *M* sodium chloride at p_H 7.2; (b) 0.4% edestin in broth at p_H 7.4.

Filtration end-point curves. The end-point curves were established for edestin dissolved in (a) 1.5 *M* sodium chloride at p_H 7.2, and (b) Hartley's broth at p_H 7.4 (see Fig. 2). The filterability of edestin was much improved by the pres-

ence of broth, but the filtration curves for edestin solutions were in general of the abnormal type, and in this respect they resembled those of serum globulin. A trace of protein in broth solution passed a $20m\mu$ membrane on one occasion, but membranes of porosities below $18m\mu$ have always retained the edestin. This filtration end-point $18m\mu$ indicates a particle diameter $6-9m\mu$, in good agreement with the value $8m\mu$ established by Svedberg and Stamm [1929]. Thus the ratio of the particle diameter, $8m\mu$, given by sedimentation analysis, to the limiting pore diameter for the complete retention of edestin is 0.44.

Egg albumin.

The egg albumin was prepared from a Schering-Kahlbaum product which originally contained globulin. After refractionation by means of half-saturated ammonium sulphate, the albumin was three times recrystallised from solution at p_H 4.7 in the presence of ammonium sulphate and then dialysed against distilled water. The final product yielded aqueous solutions which were quite colourless and contained only a very slight trace of sulphate.

The filtration end-point for egg albumin had previously been found by Elford [1933] to be $6m\mu$, a value confirmed by Bauer and Hughes [1934]. In the present study the influences of filtration conditions upon the filterability of the protein have been more extensively investigated.

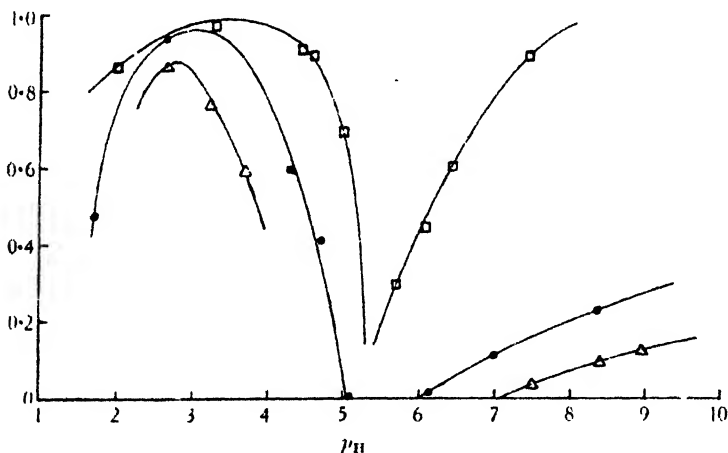


Fig. 3. Variation of u_{max} for 0.5% egg albumin solutions of different p_H values when filtered at 3 atmos. pressure through Δ 9 $m\mu$ membranes; \bullet 11 $m\mu$ membranes; \square 23 $m\mu$ membranes.

Influence of p_H . Curves showing the variation of u_{max} with p_H for three different grades of membrane are given in Fig. 3. In all cases adjustments of p_H were made by adding 0.1 *N* HCl or 0.1 *N* NaOH and the final protein concentration was kept at 0.5%. A well-defined zone of minimum filterability occurs in the region of p_H 5.0–5.5, which is slightly more alkaline than the usually accepted isoelectric point, p_H 4.6. The surface activity of the protein is maximum in this region, as shown by a minimum value in the surface tension of its solutions and a maximum degree of adsorption on collodion particles (see Fig. 5).

Influence of sodium chloride. The presence of salt in the solution may exert a profound influence upon the filtration behaviour of a protein. Egg albumin is a protein which is readily soluble in water in contrast to the relatively insoluble

globulins typified by edestin in the previous section. The influence of varying salt concentrations upon the filterability of egg albumin is shown in Fig. 4.

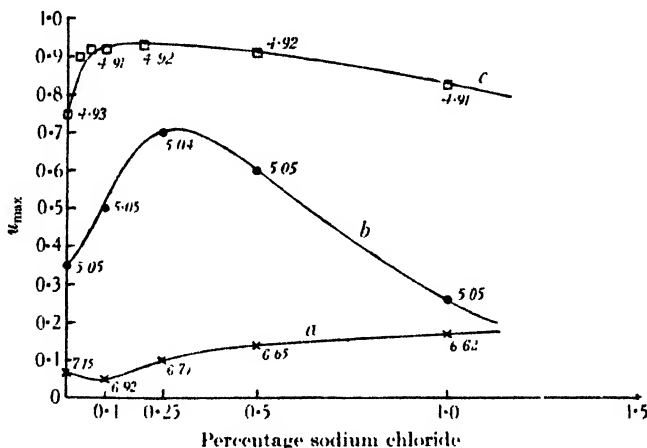


Fig. 4. Variation of u_{\max} for 0.5% egg albumin solutions containing varying amounts of NaCl. Curves "a" and "b" are for 11 $m\mu$ membranes, and curve "c" for 19 $m\mu$ membranes. The figures against the points are the respective p_H values.

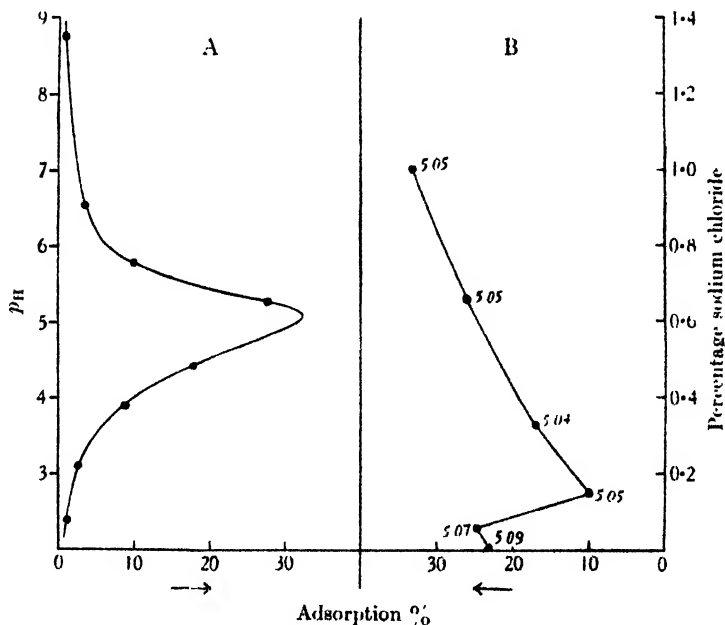


Fig. 5. Adsorption of egg albumin on collodion particles. A: from 0.5% solutions at different p_H values. B: from 0.5% solutions with different concentrations of NaCl at same p_H 5.05. The actual measured p_H values are inserted against the points in the graph.

Curves (a) and (b) give the variation of u_{\max} with the percentage concentration of NaCl for 0.5% protein solutions at p_H 7.0 and p_H 5.05 respectively, filtered through 11 $m\mu$ membranes. Curve (c) is for p_H 4.93 and more porous membranes,

19 $m\mu$. The p_H value of each solution is inserted near its appropriate point on the graph. The initial decrease in filterability shown in the early portion of curve (a) is fully accounted for by the change in p_H (cf. Fig. 3), and it is apparent that the presence of NaCl in concentrations up to 1 % slightly improves the filterability of egg albumin at p_H 7.0. Considering curve (b) for p_H 5.05 the effect of the salt concentration is here more pronounced. Initially there is a definite improvement in filterability, u_{max} increasing until the concentration of sodium chloride reaches 0.2–0.3 %, when a decrease commences and continues until for 1 % salt the value of u_{max} is slightly lower than that for the salt-free solution. The direction of the effect is similar in curve (c) but the maximum is less pronounced with the more porous membrane. Studies of the adsorption of egg albumin at p_H 5.05 on to collodion particles have demonstrated that for small additions of salt a region of minimum adsorption exists (see Fig. 5) which corresponds with the zones of maximum filterability in curve (b), Fig. 4. The change in filtration behaviour may thus be correlated with the modified surface properties of the protein in the presence of added electrolyte. Further, and largely in consequence of such modified surface properties, the state of dispersion of the colloidal protein units may be expected to be affected, particularly in more concentrated solutions.

Comparison of different media at p_H 7.2. The filterabilities of 0.5 % solutions of egg albumin in water, 1 % sodium chloride, $M/15$ phosphate buffer (Sørensen), Hartley's broth and extra-digested broth (no protein detectable), each at p_H 7.2, were compared. The values of u_{max} are shown in Table II.

Table II.

Medium	u_{max}	Remarks
Water	0.05	A.P.D. of membranes used 9 $m\mu$
1 % NaCl	0.12	
$M/15$ phosphate buffer	0.15	Filtration pressure = 3 atmos.
Extra-digested broth	0.15	
Stock Hartley's broth	0.45	

Conforming with our previous studies, Hartley's broth is seen to be the best medium for filtering egg albumin. However, considerable variation is found among different batches of broth. The value of the broth from the filtration standpoint is determined largely by the stage at which the digesting process is arrested in its preparation [Elford and Ferry, 1934]. A good broth enables as much as 75 % of the egg albumin in a 1 % solution to be obtained in the filtrate through a 9 $m\mu$ membrane [Elford, 1933], whilst in the other extreme case of the extra-digested broth containing no surface-active constituent, the percentage of protein passing is much lower, comparable with that in 1 % saline and $M/15$ phosphate buffer media—viz. 10 to 15 % only. This represents a slight improvement upon the filterability of the protein in water, although a different result might be expected at a p_H nearer the isoelectric zone (see curve (b), Fig. 4).

DISCUSSION.

The studies with the serum proteins [Elford and Ferry, 1934], haemocyanin, edestin and egg albumin, have revealed a general uniformity in filtration behaviour among the proteins, particularly in respect of the influence of medium and hydrogen ion concentration. The optimum conditions may be said to be within the limits of p_H 7 to 8, whilst of the several media investigated, Hartley's

broth has invariably facilitated filtration most. For serum albumin, egg albumin and edestin, a zone of good filterability was found in each case for aqueous solutions more acid than the isoelectric point of the protein, but the prevailing conditions of p_H were somewhat outside the p_H range of molecular stability found by Svedberg, whose sedimentation data suggest some degradation of the molecule in extreme acid solutions.

It is of particular importance that the optimum conditions for the filtration of proteins coincide with those already established for viruses and bacteriophages. This enables comparisons of their relative filterabilities and particle sizes to be made on a reliable common basis.

Elford [1933], as the result of experiments with suspensions of known particle size, concluded that under comparable optimum conditions of filtration, the particle sizes of suspensions might be deduced from the relationship $d = (\text{factor}) p$, where d = particle diameter, p = end-point pore diameter. The value of the factor adopted for the range of membrane porosities 10 to 100 $m\mu$, within which our present studies have been mainly conducted was 0.33 to 0.50. The probable particle size is thus expressed as lying between the limits $0.33p$ and $0.5p$. The data for the proteins whose molecular particle sizes have been independently determined by the method of ultracentrifugal sedimentation analysis enable a check to be made on this value of the "factor". In Table III the relevant data are summarised.

Table III.

Protein	D_s $m\mu$	p $m\mu$	D_s/p
Haemocyanin (<i>Helix</i>)	24	55	0.44
Edestin	8	18	0.44
Serum pseudoglobulin (horse)	6.9	11-12	0.57-0.63
Serum albumin (horse)	5.4	9-11	0.54-0.60
Egg albumin	4.34	6	0.72

D_s = particle diameter by sedimentation analysis. p = end-point pore diameter.

The values of D_s/p , except that for egg albumin, are seen to lie between 0.4 and 0.6, thus affording good agreement with the adopted value of the "factor", for membranes of A.P.D. greater than 10 $m\mu$. Provided that the optimum conditions for filtration do prevail when the end-point is determined, the protein data suggest that the true particle size will correspond more closely with the upper rather than the lower value deduced from the pore diameter, i.e. the true conversion factor will be nearer 0.5 than 0.33.

The closer approximation of the limiting pore size to that of the retained particle indicated by the figures for egg albumin for membranes of porosities less than 10 $m\mu$ has been previously discussed [Elford, 1933].

SUMMARY.

1. The proteins haemocyanin (*Helix*), edestin and egg albumin, like the serum proteins, animal viruses and bacteriophages, have been found to filter best when dissolved in Hartley's broth at p_H 7 to 8. The influences of various media, of p_H and of the concentration of neutral salt on filterability have been studied.

2. The filtration end-points have been established for haemocyanin (*Helix*) 55 $m\mu$ and edestin 18 $m\mu$, indicating particle sizes 18-28 $m\mu$ and 6-9 $m\mu$ respectively.

3. The results are shown to provide further confirmation of the value of the factor employed in deducing the particle size of a suspension from the porosity of the limiting membrane which just completely retains the disperse phase.

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XVII. ULTRAFILTRATION STUDIES WITH NORMAL HORSE SERUM.

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THE purpose of this investigation has been the acquisition of further evidence upon the important biological question as to the state of dispersion of the proteins in normal serum. Do the albumin and globulin fractions obtained from serum by salting-out processes exist as chemical individuals in the native state, or are they the artificially resolved constituents of a natural albumin-globulin complex? The method of ultrafiltration analysis has been applied in this study.

Previous filtration work.

The numerous references in the literature relating to ultrafiltration of various kinds of sera are mostly concerned with the distribution of inorganic salts between the colloidal and crystalloidal components in serum. The paucity of detailed investigations of the state of dispersion of the serum proteins is no doubt due largely to the lack of suitably graded membranes. Collodion sacs, although useful for the separation of colloidal and crystalloidal fractions, as in dialysis operations, have not the reproducibility and uniformity of pore size which is required when systematic analysis over a range of graded porosities is proposed. Bendien and Snapper [1933] filtered normal and pathological human sera through collodion sacs; the proportion of albumin to globulin in the ultrafiltrate was two to three times that in the original serum. This evidence that the albumin filtered more readily than the globulin made the existence of an albumin-globulin complex in serum very improbable.

EXPERIMENTAL.

The graded series of flat collodion membranes described by Elford [1931] is very suitable for such an investigation as that here considered and has been employed in all the analyses of sera described in this paper. These membranes [Elford, 1931; Elford and Ferry, 1935] are characterised by their excellent reproducibility and uniformity and the wide range of porosities available. The serum has been filtered under positive pressures of air or nitrogen employing the form of ultrafilter described by Barnard and Elford [1932]. In each experiment successive samples (each 1 ml. approx.) of filtrate have been collected in calibrated glass receiving tubes, and then from the subsequent analyses filtration curves have been constructed [*cf.* Elford and Ferry, 1934, on the filtration of serum proteins].

Analysis of filtrates.

The amounts of protein in the filtrates were determined refractometrically with a Zeiss dipping refractometer. Precipitation tests were also made, half-saturated ammonium sulphate being used to indicate the globulin, whilst

salicysulphonic acid served to indicate the albumin remaining in the solution after filtering or centrifuging off the globulin precipitate. Comparisons were made with a control series of dilutions of the unfiltered serum.

Preparation of serum.

Several different batches of normal horse serum have been studied. The blood was collected aseptically and allowed to clot spontaneously, unless otherwise stated. The serum was pipetted from the clot and centrifuged for 15 min. at 2000 r.p.m. It was then filtered through a membrane of average pore diameter (A.P.D.) 0.45μ to remove any chance contaminating organism and to furnish the serum in a condition suitable for the subsequent ultrafiltration analyses. This filtered serum was kept in a conical resistance glass flask having a tight-fitting cotton-wool plug and was stored in the cold at 0° . Before using the serum in any particular experiment the flask was placed in a water-bath at 25° for at least 20 min. so that the equilibria of the colloidal system could become readjusted for room temperature. Frequently after storage in the cold a sediment formed in the serum but this quickly dissolved on warming. The sensitiveness of colloidal equilibria to changes of temperature and salt concentration makes it very necessary to adopt a uniform treatment for all samples of serum studied in order that reliable comparisons of results may be made.

Filtration curves of normal horse serum.

When normal horse serum prepared in the manner outlined was filtered through graded membranes having progressively lower porosities an interesting series of filtration curves was obtained (Fig. 1). Membranes of A.P.D. $> 90 m\mu$

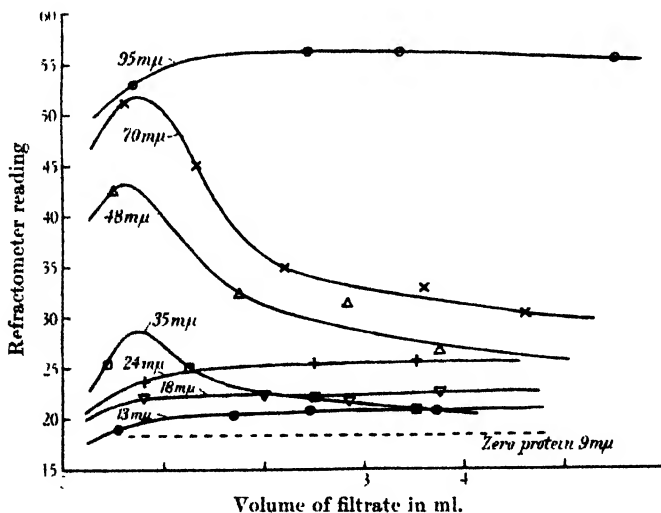


Fig. 1. Filtration curves of normal horse serum. A.P.D. values of membranes inserted against the appropriate curves.

permitted the serum to pass with undiminished protein content, and the curves were normal in type, *i.e.* when the adsorption capacity of the membranes had been satisfied the serum passed in entirety with little evidence of subsequent blocking. Over the range of porosities $80 m\mu$ to $35 m\mu$ the curves became abnormal in type,

the concentration of protein in the filtrate reaching a maximum value and then falling off rapidly as blocking of the pores set in. However, in the region of $25\text{ m}\mu$ reversion to the normal type of filtration curve occurred. These facts suggest that all the protein of normal serum may traverse pores having diameters $> 90\text{ m}\mu$ almost unhindered when the adsorption capacity has been saturated. However, with porosities $< 90\text{ m}\mu$ a portion at least of the protein has increasing difficulty in passing the protein-coated pores and the membrane becomes choked. The curves indicate that the blocking is never complete but tends towards a limiting value which still permits a small fraction of the protein to pass. It is significant that this corresponds roughly to the maximum reached in the normal filtration through even less porous membranes. These normal curves obtained with porosities $< 30\text{ m}\mu$ suggest that a fraction of the serum protein, some 20 to 30 %, is able to pass fairly readily in spite of the initial surface adsorption. We are thus led to the conclusion that in normal serum we are not dealing with a homo-disperse colloidal protein system.

End-point curve for the protein of normal horse serum.

The end-point curve for the total proteins was obtained by plotting the values of the maximum relative protein concentration (u_{max}), furnished by the filtration curves, against either the A.P.D. or the log (A.P.D.) of the respective membranes. The maximum relative concentration of protein in the filtrate yielded by any particular membrane was given by

$$u_{\text{max}} = \frac{(u_{\text{filtrate max}} - K)}{(u_{\text{serum}} - K)},$$

where

$u_{\text{filtrate max}}$ = maximum refractometer reading for the filtrate, read from the filtration curve.

u_{serum} = refractometer reading for whole serum.

$K = u_{\text{water}} + u_{\text{non-protein}}$ = refractometer reading of the filtrate from a $9\text{ m}\mu$ membrane, which is the most porous membrane to retain all the protein of serum. This value is characteristic for each particular serum.

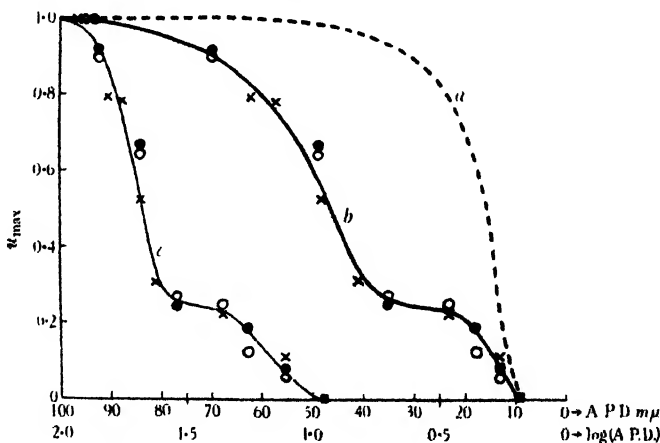


Fig. 2. Filtration end-point curves for total proteins of normal horse serum, corresponding to three independent series of observations on different batches of horse serum. Zero protein given by $9\text{ m}\mu$ filtrate in each case.

Curve (a) anticipated curve for monodisperse system; curve (b) established curve for normal horse serum, u_{max} plotted against A.P.D.; curve (c) corresponding curve to (b) for u_{max} plotted against log (A.P.D.).

The end-point curve based on three independent series of observations for different batches of normal horse sera is given in Fig. 2, plotted in curve *b* as u_{\max} against A.P.D. and in curve *c* as u_{\max} against \log A.P.D. It is seen that membranes of A.P.D. $< 10 m\mu$ retained all the protein. This is significant, for it agrees with the end-points found by Elford and Ferry [1934] for serum albumin and pseudoglobulin separately, these being 9 to $10 m\mu$ and 11 to $12 m\mu$ respectively. This strongly suggests that these proteins do exist independently in serum, for were they present only in an associated form a rather higher end-point value indicating a greater particle size would be expected. A further interesting and very suggestive feature characterises the end-point curve. A definite flattening of the curve, varying slightly among different batches of serum, is situated in the region of porosity 20 to $30 m\mu$. The curve appears to be the resultant for a system containing protein in two broad states of dispersion and cannot be explained purely in terms of variable adsorption effects. Without analysing the interpretation further at this juncture it will be helpful to adopt as a provisional hypothesis that the section of the end-point curve in the region of porosity 30 to $90 m\mu$ corresponds to the filtration of a complex protein, whilst the portion from 20 to $10 m\mu$ corresponds to the filtration of molecular protein.

Filtration of diluted serum.

The filtration and end-point curves were determined for serum which had been diluted in physiological saline and also in Hartley's broth at p_H 7.6. The proportions in each case were one part of serum to nine parts by volume of the diluent. The results given in the end-point curves of Fig. 3 show that dilution

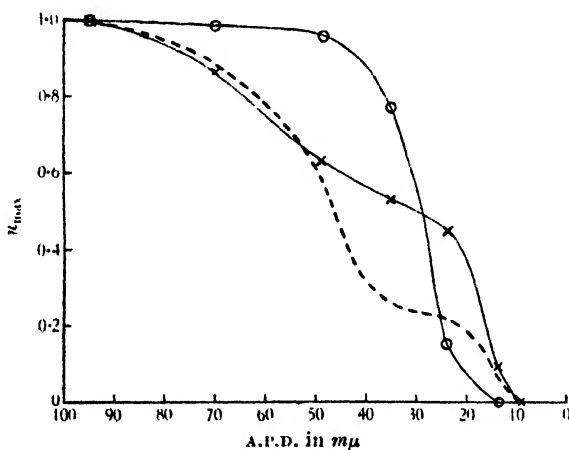


Fig. 3. Filtration end-point curves for diluted serum compared with that for whole serum. Dotted curve, whole serum; \circ , serum diluted 1 : 9 in saline; \times , serum diluted 1 : 9 in broth.

has a pronounced effect upon the filterability of the serum proteins. Whilst the end-point for the complete retention of all the protein remains essentially what it was for undiluted serum, the form of the curve for the saline dilution now corresponds to that of a homodisperse system and this is supported by the fact that the filtration curves were all normal in type. The filterability of the broth-serum system suggests that in this case the proportion of complex protein has been reduced and that of the molecular fraction increased compared with undiluted serum. These results indicate that the postulated complex protein readily tends

to dissociate when the system is diluted in an appropriate medium, saline being much more favourable than broth. Comparison of the curves for saline and broth dilutions shows that the end-point for the saline system is slightly higher than that for the broth system, being 13 $m\mu$ instead of 9 to 10 $m\mu$. This is probably to be explained by the increased adsorption of the saline system [cf. filterability of proteins in different media, Elford and Ferry, 1934].

Filtration of aged serum.

Serum stored at 0° in the manner already indicated for periods of from 6 to 20 months exhibited definite differences in filtration behaviour compared with fresh serum. The end-point curves of Fig. 4 illustrate how the filtration of serum

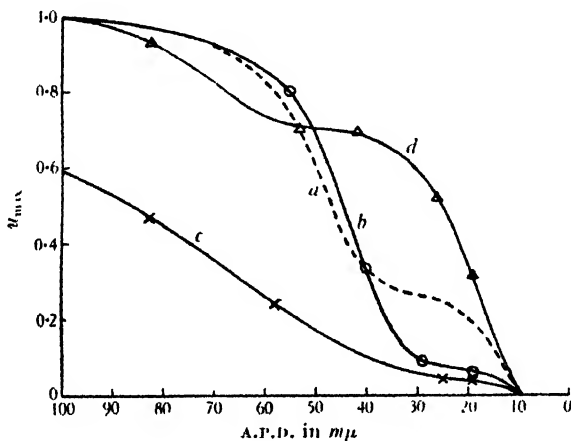


Fig. 4. Filtration end-point curves for aged serum. (a) Dotted curve, normal serum about 2 weeks old. (b) \circ , normal serum 15 months old. (c) \times , normal serum 22 months old. (d) \triangle , dilution 1 : 9 in broth of the 22 months-old serum.

proteins depends upon the age of the serum. Comparing curve (a) for normal serum about 2 weeks old with curve (b) for the same serum after 15 months at 0° it appears that aggregation of the protein has proceeded further and that the proportion of molecularly dispersed protein has decreased. Curve (c) for a still older serum (22 months at 0°), but of a different batch from (a) and (b), further emphasises the effect. The gradual slope of the curve indicating partial retention of protein over a greater range of porosity than for fresh serum suggests that particles of varying sizes have been formed through aggregation of protein units. In order to ascertain whether this secondary aggregation was also of a reversible type some of the 22 months-old serum was diluted 1 : 9 in broth and filtered. The resulting curve (d) shows again that the aggregates have been resolved, for it closely resembles the curve for fresh serum similarly diluted.

Filtration of Type I pneumococcus antiserum.

Filtration experiments were made with some Type I antipneumococcus horse serum. Unfortunately freshly collected serum was not available, and the preparation used was an oxalated serum 30 months old and had been stored after filtration without preservative in a waxed stoppered bottle. The potencies of the undiluted antiserum and of the filtrates were estimated by the optimum

proportions test. We are much indebted to Dr Wilson Smith for his advice concerning the technique of this method. It was estimated that 75 % of the antibody passed a 140 $m\mu$ membrane, rather less than 1 % passed an 80 $m\mu$ membrane, whilst the filtrate from a 54 $m\mu$ membrane showed no trace of antibody activity. Although these experiments are not strictly comparable with those on the normal sera previously described, since no adequate control on the treatment to which the serum had been subjected was possible, nevertheless the result suggests very strongly that the antibody activity is associated with the larger complex protein in the serum. Further, the possibility is presented of concentrating the antibody above a relatively porous membrane.

DISCUSSION.

The complete retention of the proteins of normal horse serum by all membranes of A.P.D. < 10 $m\mu$, whether the serum is whole or diluted, fresh or old, indicates that the smallest unit of the protein has a particle size of about 5 $m\mu$ [Elford, 1933]. The limiting porosity coincides with the end-point found for horse serum albumin when solutions of the crystallised protein are studied, and the particle size compares well with the findings of Svedberg and Sjogren [1930] in their centrifugal studies with this protein. Within narrow limits of porosity, 10 to 13 $m\mu$, filtrates of normal serum have frequently given positive tests for albumin but no evidence of globulin. This again accords well with the observations on individual proteins [Elford and Ferry, 1934], the end-point for pseudoglobulin having been found to be slightly higher than that for serum albumin, viz. 11 to 12 $m\mu$ as compared with 9 to 10 $m\mu$. These facts have led us to the conclusion that the albumin and pseudoglobulin fractions of serum do co-exist as individual molecular species in the native state. However, the observations recorded in the foregoing paragraphs would further suggest that the proteins of serum are not homodisperse. Experience with the membranes used in this study has led us to expect an end-point curve of the form (a) in Fig. 2 for a homodisperse suspension. The experimentally established curve (b), Fig. 2, for undiluted serum suggests that the native protein exists broadly in two different states of aggregation, i.e. besides the molecularly dispersed albumin and pseudoglobulin whose end-points are quite close together, there is an additional protein unit having a particle size approximately twice that of molecular serum albumin. Precipitation tests and values of the albumin globulin ratio, given by nitrogen estimations for which a modification of the method of Howe [1921] was used, have indicated that the more readily retained fraction of the serum protein consists principally of globulin, whilst the major portion of the more filterable fraction passing membranes below 30 $m\mu$ is albumin. The fact that only 20 to 30 % of the total albumin is accounted for in these filtrates, and even a smaller proportion of the total globulin, would be explained either by the presence of associated forms of these proteins or by a statistical effect of sieving, through globulin fraction. Maybe both these factors operate. Studies, in continuation of those recorded here, on the filtration of the euglobulin fraction of horse serum by one of us [Grabar, 1934] have shown that this protein is very definitely less filterable than either serum albumin or pseudoglobulin. Also Elford and Ferry [1934] found that, in concentrations comparable with that of the protein of serum, solutions of the individual proteins, more particularly pseudoglobulin, filtered less readily than did solutions containing only 0.5 % protein, suggesting some degree of association in higher concentrations. It seems probable therefore

that the less filterable fraction of the protein in normal serum is an associated complex of molecular protein, predominantly globulin, maybe the euglobulin. We have not followed the distribution of lipoid, but in view of the observations of Chick [1914] on the phosphorus content of the various protein fractions of serum the possibility that lipoid may also be associated with the complex must not be overlooked. The filtration experiments with diluted serum indicate strongly that the associated protein can dissociate readily under suitable conditions with the result that the system then behaves as might be expected of a mixture of the molecular proteins.

The observations on the filterability of the type I pneumococcus antiserum (horse) are in keeping with the above interpretation, since the antibody found in our experiments to be associated with the more readily retained fraction of the serum is well known to be contained in the globulin portion.

Recent ultracentrifugal studies have furnished several extremely significant facts regarding the molecular complexity of the proteins of serum. Thus Mutzenbecher [1933] found that the albumin and globulin sedimented at characteristic rates in serum, and concluded that the proteins existed as separate individuals in the native state. There was evidence that the globulin was not monodisperse. Extended studies of both normal and pathological sera by McFarlane [1935] revealed a more complex state of affairs. Normal serum was found to contain two components possessing sedimentation constants comparable with those of serum albumin and serum globulin. The lighter of these fractions however was found not to be homogeneous, but to consist of two substances, one of which (*X* substance) had a molecular weight only slightly greater than that of serum albumin, which was in the same fraction. A further interesting effect was found by McFarlane, *viz.* that on dilution the proportion of globulin increases whilst that of the two lighter constituents decreases. It becomes necessary then to postulate an equilibrium between albumin and globulin in which the compound product is of intermediate molecular weight, but rather nearer to that of albumin. The evidence at present available is insufficient to permit the formulation of the type of interaction responsible for this.

It will be seen that there is in general a large measure of agreement between the filtration results and those of sedimentation analysis. The protein found to accompany the albumin in filtrates furnished by membranes of A.P.D. $< 30\text{ m}\mu$ which we have found to behave in precipitation tests as globulin and in its filtration end-point as pseudoglobulin, may correspond to McFarlane's *X* component. The larger protein fraction which we have regarded as a globulin aggregate, possibly with lipoid and some albumin loosely associated, would correspond to the more readily sedimented globulin fraction.

Hartley [1914] presented analytical evidence that serum albumin differs chemically from serum globulin, both the eu- and pseudo-fractions of which were identical. The physical evidence of the present study supports Hartley's conclusions, for undoubtedly the albumin and globulin behave as distinct individuals in serum, the former existing mainly in the molecularly disperse condition whilst the globulin appears to be partly in an associated form and partly molecularly dispersed.

SUMMARY.

The analysis of the state of aggregation of the proteins in normal horse serum by the method of ultrafiltration through graded collodion membranes has indicated (1) that the simplest protein units present correspond in particle size to molecular serum albumin and pseudoglobulin as studied individually in

dilute solutions of the pure proteins, and (2) that in addition to the molecularly dispersed protein there is a fraction more readily retained by membranes and in particle size about twice that of serum albumin. This is essentially a globulin aggregate, maybe with some lipoid and albumin loosely associated, and in view of its poor filterability it may correspond with the euglobulin fraction of serum. The associated globulin complex appears to dissociate readily under favourable conditions, particularly upon dilution, when the filtration behaviour of the serum proteins resembles that of a mixture of the molecularly dispersed individual constituents.

The antibody of an aged sample of type I pneumococcus antiserum was retained by membranes simultaneously with the more complex globulin fraction of the serum protein.

Two of us, P. G. (Strasbourg) and W. F. (Heidelberg), desire to express our thanks to the Rockefeller Foundation and to the Medical Research Council for the facilities afforded enabling us to participate in these studies.

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XVIII. THE FATTY ACIDS OF PHRENOSIN AND KERASIN.¹

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INTRODUCTION.

THE constitution of the fatty acid components of the two cerebrosides phrenosin and kersin has been the subject of many investigations since their first separation by Thudichum, and a review of the work of Thierfelder, Levene, Rosenheim, Klenk, *etc.* is given in the two monographs by MacLean and MacLean [1926] and Thierfelder and Klenk [1930], which cover the literature to the date of their respective publications. During the last five years, however, doubt has arisen as to the constitution of phrenosinic acid, and to appreciate the position it will be necessary to recapitulate briefly certain of the earlier researches, for it is out of them that the present dispute has arisen.

Thierfelder was the first to show that Thudichum's "neurostearic" acid was a hydroxy-acid of the apparent formula $C_{25}H_{50}O_3$, whilst Levene and Jacobs [1912] suggested that it was α -hydroxypentacosanoic acid from evidence based on its oxidation to what they then considered to be *n*-tetracosanoic acid (M.P. 81°). A synthesis of (racemic) α -hydroxy-*n*-pentacosanoic acid by Brigl [1915] however led to inconclusive results. Meanwhile Levene and West [1914] modified their former view, and claimed that the acid formed on oxidation was "lignoceric" acid $C_{24}H_{48}O_2$ with a branched chain and not the normal acid, so that phrenosinic acid itself had a branched chain. Additional evidence was furnished by Levene and Taylor [1922], who synthesised apparently identical samples of α -hydroxyisopentacosanoic acid from their "lignoceric" acid prepared from phrenosinic acid and the naturally occurring "lignoceric" acid of arachis (peanut) oil. In assessing the validity of this evidence it is to be remembered that the composite nature of "lignoceric" acid was not apparent at that time, so the fact that all the products made from it were complex mixtures was not realised.

The first suggestion that phrenosinic acid was not $C_{25}H_{50}O_3$ but $C_{24}H_{48}O_3$ was made by Klenk [1928, 1], who claimed that the main product of oxidation was *n*-tricosanoic acid, M.P. 78.5° and mol. wt. 354. Further, hydrogenation of α -hydroxynervonic acid, in which the α -position of the hydroxyl group had been

¹ The nomenclature used in this paper is that suggested by Posner and Gies [1906] and Rosenheim [1909; 1914], which was adopted by MacLean and MacLean [1926]; phrenosin = Thierfelder's "cerebron" and phrenosinic acid = Thudichum's "neurostearic" acid = Thierfelder's "cerebronic" acid. We are in agreement with the present view of Dr Rosenheim that since Thierfelder himself admitted before his death that his "cerebron" and Thudichum's phrenosin are one and the same substance [cf. Thierfelder and Klenk, 1930, p. 4] it seems only fair to Thudichum's memory that his nomenclature should be retained and the names "cerebron" and "cerebronic" acid be abandoned.

fixed by degradation, gave phrenosinic acid: the latter acid must therefore also be an α -hydroxy-acid, *i.e.* α -hydroxy-*n*-tetracosanoic acid. The view was disputed by Levene and Taylor [1928], who rigidly purified some earlier samples of phrenosin by repeated crystallisation from various solvents and obtained from the phrenosinic acid by oxidation a fatty acid, m.p. 79–80°, which had mol. wt. 367 and appeared to be “lignoceric” acid. A year later Taylor and Levene [1929] returned to the subject and reported that a new sample of phrenosinic acid, prepared from some phrenosin whose preparation was not described, had given on oxidation fatty acids of distinctly lower mol. wt. than before, and from them a tricosanoic acid, m.p. 77·8–78·6° mol. wt. 356, thought to be identical with that of Klenk, was prepared. Repeated fractional distillation of this acid, however, gave a low-boiling fraction, m.p. 74–75°, which had mol. wt. 341, corresponding to docosanoic acid, and higher-boiling fractions, one of which, m.p. 78·5–79·5°, had mol. wt. 367, corresponding to tetracosanoic (“lignoceric”) acid. They therefore suggested that phrenosinic acid was a mixture. Klenk and Diebold [1933] emphatically refuted this claim of Taylor and Levene. They again oxidised a large sample of phrenosinic acid, rigidly fractionated the resulting acids as esters in a still of the type designed by Jantzen and Tiedcke [1930] and claimed a yield of 85% of *n*-tricosanoic acid, m.p. 79·5°, which gave no depression when melted with a sample of synthetic acid, m.p. 79·5–80°. The later publications of these authors [Levene and Heyman, 1933; Taylor and Levene, 1933; Levene and Yang, 1933; Klenk, 1934] merely emphasise their respective points of view.

Our experience in unravelling the constitution of the mixed fatty acids and alcohols of waxes [Chibnall *et al.* 1934] suggested to us that these conflicting views might well be reconciled if it be assumed that phrenosinic and “lignoceric” acids were not chemical entities, but were each of them a mixture of near homologues. Dr Rosenheim very kindly offered to place at our disposal for an investigation on these lines samples of highly purified phrenosin and kersin prepared by him many years ago. The results were that the phrenosinic acid from the phrenosin was found to be a mixture of α -hydroxy-*n*-hexacosanoic and α -hydroxy-*n*-tetracosanoic acids, whilst the “lignoceric” acid from the kersin, like the similar acid frequently isolated from other natural sources, is a mixture of *n*-hexacosanoic, *n*-tetracosanoic and *n*-docosanoic acids.

EXPERIMENTAL.

Melting points. The melting points recorded in this paper were obtained by the method described by Piper *et al.* [1931], in which the temperature of the bath is raised very slowly. Rapid heating according to the method of Levene [*cf.* Levene and Taylor, 1922] in which the temperature of the bath is raised 6–7° per min., gives a melting-point about 1° higher, *e.g.* a sample of phrenosinic acid which we record as melting at 102–102·5° would melt at 102·5–103·5° by Levene's method.

1. *Phrenosinic acid.*

Preparation and general properties. The sample of phrenosin was of the same degree of purity as those described by Rosenheim [1914], and was reported by him to be practically free from phosphorus, to have the correct rotation and to show the absence of kersin in the selenite plate test. It was hydrolysed in the usual way with 10% methyl alcoholic H_2SO_4 , the phrenosinic acid separated as the ester and purified through the magnesium soap [Thierfelder, 1913; Klenk, 1927]. It melted at 101·3–101·5° and crystallised from alcohol in the well-known “cauliflower-like” masses described by Thudichum, which are made up of

irregular sphaero-crystals. Attempts to obtain crystals from warm acetone were unsuccessful, as the product gelled. X-ray analysis gave a very poor plate (Series No. B 390) showing three diffuse orders. Further purification was effected in the following way. The material (1.15 g.) was dissolved in pyridine (20 ml.), which was then poured into water. The aqueous solution was extracted twice with ether and then acidified with excess of hydrochloric acid. The precipitated phrenosinic acid was extracted with ether, the ethereal solution washed until free from mineral acid, filtered to remove a slight suspension of insoluble material and the solvent removed. The residue was crystallised from 30 ml. of alcohol at 0°, the product taken up in the same volume of warm acetone and filtered from a small insoluble residue. On cooling the acetone solution to 25° the phrenosinic acid separated in the usual "cauliflower-like" masses, which melted sharply at 102.3–102.6° and set in long needles at 101.8°. (Found: C, 75.54, 75.40; H, 12.47, 12.63%; mol. wt. by titration 396. $C_{25}H_{50}O_3$ requires C, 75.30; H, 12.65%, mol. wt. 398.4.) For a 6.01% solution in pyridine the observed rotation was $\alpha_D^{25} + 0.20^\circ$ whence $[\alpha]_D^{25} = +3.33^\circ$. X-ray analysis (Series B 405) gave a better plate with 5 orders measuring 54 Å., but the lines were still diffuse.

Attempts to prepare larger crystals more suitable for X-ray analysis by using different solvents and conditions were not very successful, and the best photograph was finally obtained from material crystallised from 85% ethyl alcohol. The spacings obtained (Series No. B 411) were 55.4 Å. and 50.9 Å., but in the absence at this stage in our investigation of data concerning the crystal spacings of α -hydroxy-acids we could make no suggestion of chain length and could only infer from the poor quality of the plate that the phrenosinic acid, if a chemical entity, was heavily contaminated with some other acid.

To effect further purification the acid was acetylated with acetyl chloride in the usual way, and the product, which was freely soluble in cold light petroleum, separated from that solvent on evaporation in clusters of microscopic broken needles which melted at 66–66.5°. (Found: C, 73.69, 73.61; H, 11.96, 11.79; OC.(CH₃) 9.6. $C_{27}H_{52}O_4$ requires C, 73.55; H, 11.9; OC.(CH₃) 9.7%.) The acetyl value was determined by the micro-method of Kogl and Postowsky [1924]. It was extensively fractionated without change of m.p. and no evidence for the presence of "lignoceric" acid was obtained, showing that the phrenosinic acid was not contaminated with this acid. Acetylation therefore suggested that if phrenosinic acid were a mixture the components were all hydroxy-acids. The X-ray photograph of the acetyl derivative was extremely poor and uninterpretable.

Study of a series of synthetic i- α -hydroxy-n-fatty acids. Unlike Levene and Klenk, who have both handled relatively large amounts of phrenosinic acid during the course of their investigations, we had at our disposal only about 4 g. and were therefore obliged to proceed with very great caution. In our previous work on the constitution of the wax fatty acids success was only achieved after careful study of the physical properties and X-ray data of the whole series of higher *n*-fatty acids and we felt that some such study would amply repay us in the present case. Synthesis of α -hydroxy-acids of chain length from (say) C_{20} to C_{26} was however not feasible, and we were fortunate in being able to obtain from Dr N. K. Adam samples of the inactive acids from C_{13} to C_{18} prepared by Le Sueur [1905].¹ These products had been synthesised at a time when the criteria necessary for judging the purity of their *n*-fatty acid precursors were

¹ A sample of α -hydroxyheptacosanoic acid prepared by Le Sueur and Withers [1915], by an indirect method from erucic acid, gave a very poor photograph. The melting point (91–92°) was too low for the pure acid.

still ill-defined, and it is a great tribute to the experimental skill of the late Dr Le Sueur that they stand up so remarkably well to the exacting requirements of modern X-ray analysis, for all of them, after appropriate crystallisation from acetone, gave excellent X-ray photographs.

The melting points, in agreement with Le Sueur's, are given in Table I, and as he observed, they lie on one smooth curve and show no odd-even alternation characteristic of the *n*-fatty acids. We have prepared all the acetyl derivatives

Table I. *Melting points and long crystal spacings of a series of α -hydroxy-*n*-fatty acids.*

No. of carbon atoms	M.P. (°C.)	Series no.	Crystal spacings in Å.			Acetyl derivative M.P. °C.	Difference between M.P. of acid and its acetyl derivative °C.
			Pressed (P) or melted (M) layer	A	B	C	
13	78	B 409	P	34.55	—	—	—
14	81.5–81.7	B 408	(P) (M)	— *38.7	33.7 33.75	31.8	56–56.5 —
15	84.5–84.7	B 396	(P) (M)	39.5 39.1	—	—	49.5–50 —
16	86.9–87.2	B 389	(P) (M)	— 12.3	38.0 38.05	—	63.2–63.5 —
17	89.3–89.6	B 395	P	44.6	—	—	59.3–59.5
18	91.5–91.7	B 394	(P) (M)	— —	42.25 42.3	39.0 40.2	70.1–70.3 —
Phrenosinic acid	102.3–102.6	B 411	(P) (M)	— —	55.1 50.2	50.8	66.5 —
70° ₀ C ₁₅ + 30° ₀ C ₁₇	—	B 399	P	12.6	—	—	—
70° ₀ C ₁₆ + 30° ₀ C ₁₈	—	B 400	P	—	39.7	37.6	54.5
40° ₀ C ₁₁ + 40° ₀ C ₁₆ + 20° ₀ C ₁₈	74.6	—	—	—	—	—	39.0

* Only first order observed.

and unexpectedly in this case there is a pronounced odd-even alternation in the M.P. curve. This is reflected in the figures given in the last column of Table I, showing the differences between the M.P. of the acids and their corresponding acetyl derivatives: that of the C₁₈ acid is 23.7°, of the C₁₇ acid 30.1° and of the C₁₆ acid 21.4°. Extrapolating from these results the C₂₄ and C₂₅ acids will melt at about 101° and 103°, and their acetyl derivatives about 83 and 85° respectively, giving a difference of 16–18°. (The synthetic C₂₅ acid of Brigl [1915] melted at 102–104°; no acetyl derivative was recorded.) These figures are in sharp contrast to those of phrenosinic acid and its acetyl derivative, with a difference of 36.5° as shown in Table I. It is true that this latter acid is optically active, so that a direct comparison is not warranted, yet the evidence certainly casts doubt on Klenk's contention that it is α -hydroxy-*n*-tetracosanoic acid, for in that case the optically active isomeride would melt at the most only 1–2° higher than the inactive acid, whilst its acetyl derivative would melt 17–18° lower. A series of mixed inactive acids and of the corresponding acetates was then prepared. The melting points of a few examples are recorded in Table I; these certainly lend support to the impression gained from the X-ray study of phrenosinic acid, that it is a mixture of hydroxy-acids. Before discussing the X-ray data given in Table I, which lead to a similar conclusion, it will be convenient to consider whether phrenosinic acid is a mixture which might contain β - or γ -hydroxy-acids.

Klenk [1927] showed that when a boiling alcoholic solution of magnesium acetate was added to a boiling alcoholic solution of phrenosinic acid the magnesium soap was immediately precipitated and he made use of this observation to separate this acid from "lignoceric" acid. The property is shared by the lower α -hydroxy-acids of Le Sueur, but γ -hydroxystearic acid [Clutterbuck, 1924], which has been tested, gives no insoluble magnesium soap under these conditions, so that if phrenosin on hydrolysis gives any γ -hydroxy-acid or its lactone these would, during the magnesium soap separation, pass into the "lignoceric" acid filtrate, for both of them would be soluble in boiling alcohol. The presence of a γ -hydroxy-acid or its lactone in phrenosinic acid can therefore be definitely excluded.

Brigl [1915] obtained the first direct evidence for the presence of an α -hydroxyl group in phrenosinic acid by preparing the chloralide; no yield was quoted however so that if the acid be a mixture the presence of a β -hydroxyl group is not thereby excluded. We have sought for evidence of the latter by very cautious oxidation. Under these conditions the α -hydroxy-acid should give the ($n-1$) aldehyde and its corresponding acid, whilst the β -hydroxy-acid should give the β -keto-acid, the methylketone and the ($n-2$) fatty acid. The oxidation gave 21.5% of neutral material, and a parallel oxidation of α -hydroxypalmitic acid gave a somewhat smaller yield. The material from the phrenosinic acid, on recrystallisation from alcohol, melted at 53–53.5°, and had all the properties of the lower (C_{13} , C_{17}) aldehydes described by Le Sueur [1905], *viz.*, it was readily soluble in all ordinary organic solvents in the cold, reduced permanganate slowly in the cold, gave a semicarbazone which crystallised in needles melting at 107–108° and was fairly readily polymerised in moist ether to give a product insoluble in cold ether and alcohol. Extrapolation of Le Sueur's data shows that n -tricosanaldehyde would melt at about 52–54° and its semicarbazone at about 109–110°, whereas the known data for methylnonadecanylketone suggest that methylheneicosanylketone would melt at about 67° and its semicarbazone at 130°. The evidence shows clearly that the hydroxy-group in phrenosinic acid is largely, if not entirely, in the α -position, in agreement with Klenk [1928, 1].

Since the series of α -hydroxy-acids given in Table I does not show alternation of m.p., the acids should adopt a crystalline modification characterised by a vertical molecule in the neighbourhood of the m.p. for both odd- and even-numbered chains [Malkin, 1931]. The odd-numbered acids examined were found to occur only in this A modification which was also found for α -hydroxypalmitic acid in a layer that had been very quickly cooled from the melt. A single, diffuse but measurable line for a similar layer of the α -hydroxymyristic acid gave a value suggesting that this modification was present, but we were unable to detect it with certainty in the case of α -hydroxystearic acid. A photograph taken with this acid on a heating stage at a temperature that kept it partly liquid did show a very weak long spacing, but the lines were too faint to measure with certainty. The modification can presumably exist only very near to the m.p. of this acid and must have a very small range of stability. Of the B and C forms observed for the even-chain acids B is the stable modification.

These acids in the pure form give very good reflections. The orders are recorded up to the extreme angle of rock, and the intensity distribution differs characteristically from that of a n -acid. As usual the mixtures give poorer reflections and more diffuse lines, and frequently not enough orders of reflection are registered to establish the intensity distribution characteristic of the series. Phrenosinic acid gave a photograph characteristic of a mixture and since it crystallises in two forms it cannot be a mixture of odd-number acids. The mea-

sured spacings 55.4 Å. and 50.8 Å. agree tolerably well with the extrapolated data 55.5 Å. and 51.5 Å. for C_{24} hydroxy-acid, but not with the spacings for C_{23} or C_{25} which would be about 60 and 65 respectively. Thus the crystallographic measurements suggest a mixture of even-number hydroxy-acids of mean mol. wt. corresponding closely to C_{24} .

The fatty acids obtained by oxidation of phrenosinic acid. Two samples of the acid were oxidised, the subsequent treatment of the oxidation product differing in each case.

(1) 0.6 g. of phrenosinic acid was dissolved in 80 ml. of glacial acetic acid at 60° and treated at that temperature with 0.4 g. of CrO_3 in 20 ml. of the same solvent added in small amounts during the course of 15 min. Excess of water was then added and the precipitated fatty acid filtered off and thoroughly washed. It was then dissolved in 60 ml. of boiling alcohol and treated with a hot alcoholic solution of magnesium acetate to precipitate the major part of any unchanged phrenosinic acid. This was filtered off and the fatty acid remaining in the alcoholic filtrate recovered in the usual way. The product (0.45 g.) was then treated with boiling acetyl chloride for 2½ hours, the mixture poured into excess of chilled water and the precipitated material filtered off. This was dissolved in ether, well washed with water, the solvent removed and the residue crystallised from acetone at room temperature to free it from any acetylphrenosinic acid. The resulting material (0.35 g.) was then saponified with 5% alcoholic NaOH for 1 hour to reconvert any acid anhydride formed in the previous operation, the sodium soap was well washed with ether and the acid recovered in the usual way. Two crystallisations from acetone at 37° gave fraction 1 (0.17 g.), m.p. 77.4–77.6°. The mother-liquors gave crystalline fractions with mol. wt. between 360 and 390, suggesting that the treatment with acetyl chloride had led to more extensive anhydride formation than was expected, and the products were discarded.

(2) Another 0.6 g. of phrenosinic acid was oxidised under the same conditions as before. Excess of water was then added and the precipitated fatty acid filtered off and thoroughly washed with water. The dry product (0.54 g.) was dissolved in ether, which was filtered to remove a small insoluble residue. An alcoholic solution of sodium ethoxide was then cautiously added until precipitation of the sodium soap was complete. This was centrifuged and washed repeatedly with ether; the fatty acid was recovered in the usual way and crystallised from acetone at 0°, m.p. 75.2–75.4°. This acid (0.43 g.) was then fractionated by the method of Klenk [1928, 1]. It was dissolved in 10 ml. of boiling methyl alcohol and treated successively with 0.3 ml., 0.7 ml. and 0.7 ml. of boiling methyl alcoholic lithium acetate, the precipitated soap in each case being filtered off hot. The first precipitate (27 mg.) was refluxed with 10 ml. of boiling methyl alcohol for 6 hours; solution was then complete, showing that very little unchanged phrenosinic acid was present. The recovered acid (10 mg.) melted at 75.4–75.6° (fraction 2). The second (0.18 g.) and the third (0.14 g.) samples of soap were converted into the free acids, which were crystallised from 10 ml. of acetone at 0° (0.15 g., m.p. 76.1–76.3°; 0.12 g., m.p. 75.6–75.8°). To obtain good crystals for X-ray analysis both acids were recrystallised slowly from acetone at 25° to give fraction 3 (95 mg.) and fraction 4 (80 mg.) respectively. The filtrate obtained after removal of the lithium soaps mentioned above gave no further precipitate on the addition of alcoholic lithium acetate, and on treatment gave 18 mg. of acid (fraction 5).

Fractions of fatty acids obtained by oxidation of phrenosinic acid.

Fraction	M.P.	C %	H %	Mol. wt. by titration
1	77.4-77.6	78.0	13.1	353
2	75.4-75.6	77.7	13.0	—
3	76.5-76.7	78.1	13.3	363
4	76.8-77.0	78.0	13.2	360
5	73.1-73.3	77.3	12.5	—

Fractions 1, 3 and 4, which represent the main bulk of the oxidised material in the two cases, have been further investigated in some detail. All three crystallised in fine plates which, under the microscope, were seen to consist of very small rhombs with sharp angles and straight cleavage edges, suggesting that they were either pure *n*-fatty acids or relatively simple mixtures.

X-ray analysis of the fatty acid fractions. From the mixed m.p. curves of Fig. 1 a list is made of all the mixtures which have melting-points close to that of the fraction to be analysed. From this list those mixtures which have mol. wt.

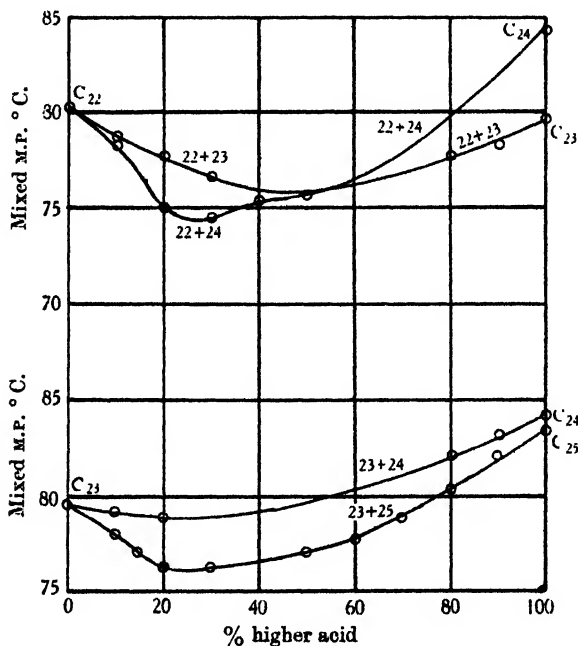


Fig. 1.

within ± 5 of that of the unknown mixture are transferred to the first column of Table II. The other columns contain the remaining relevant information. From an inspection of the table it is usually possible to find a binary mixture which is a good fit throughout. The spacings are correct to $\pm 0.5 \text{ \AA}$. Suggestions as to whether one is dealing with odd- or even-number mixtures are often obtained from an inspection of the intensity distribution in the photographs, for the even orders of reflection are usually absent from the pictures of the odd-number mixtures.

In the case of fraction 1 only one of the possible comparison mixtures (85% C₂₃ + 15% C₂₅) has two spacings which differ by about 5.0 \AA , but the m.p. of this

Table II. *Melting points and long crystal spacings of the fatty acid fractions obtained by oxidation of phrenosinic acid, together with certain standards for comparison.*

Substance	Mol. wt.	M.P. (°C.)	Mixed M.P. of fraction with comparison mixture (1:1)	Long crystal spacings in Å.			Remarks
				Series no.	Pressed	Melted	
Fraction 1	353	77.4-77.6	—	B 402	57.1	52.0	No even orders
90% C ₂₃ + 10% C ₂₅	357.2	77.8-78.0	77.5-77.7	B 412	55.7	52.1	"
85% C ₂₃ + 15% C ₂₅	358.6	76.9-77.1	77.0-77.2	B 438	57.1	52.1	"
85% C ₂₃ + 65% C ₂₄	358.5	77.3-77.6	77.0-77.3	Extra- polated	57.5	51.0	Even orders would appear
20% C ₂₃ + 80% C ₂₅	351.6	77.5-77.7	77.2-77.4	B 428	53.3	51.3	Even orders appear
10% C ₂₃ + 90% C ₂₅	353	77.7-78.0	77.5-77.7	B 430	53.3	51.6	No even orders
Fraction 3	363	76.5-76.7	—	B 441	59.2 52.6	52.3	"
80% C ₂₃ + 20% C ₂₅	360	76.3-76.5	76.4-76.6	B 413	60.9 57.45	57.1	"
Fraction 3a	—	77.0-77.2	—	B 447	57.6 53	51.7	"
85% C ₂₃ + 15% C ₂₅	358.5	76.9-77.1	76.0-77.2	B 438	57.1	52.1	"
Fraction 5	360	76.8-77.0	—	B 445	57.3	52.4	"
85% C ₂₃ + 15% C ₂₅	358.6	76.9-77.1	76.8-77.0	B 438	57.1	52.1	"
40% C ₂₃ + 60% C ₂₄	357.2	77.0-77.2	76.4-76.6	Extra- polated	57	54	Even orders would appear

mixture is a little low. The mixed M.P. however is between the two respective values, and we suggest that the mixture (87% C₂₃ + 13% C₂₅) melting at 77.4° and having spacings about 56.5 Å. and 52 Å. is a rather better fit, though the upper spacing is definitely too low. It will be noticed that the spacings only correspond at all well with those of mixed odd-number acids, and that for a range of mixtures of these acids containing 0-30% of the longer component the steep rise of the B curve allows a very accurate assessment of the proportions of a simple binary mixture. It seems probable therefore that there is slight contamination of this fraction by a third acid, probably C₂₁, and that the proportions of the main bulk of the mixture are about 87% C₂₃ + 13% C₂₅.

Only one synthetic binary mixture corresponds at all for both melting point and mol. wt. with fraction 3. Both the fraction and the mixture have the long A spacing, but their behaviour on melting differs. The appearance of the low spacing (52 Å.) on melting corresponds to less than 20% of the longer chain, whilst the A spacing suggests at least 20% of this chain, and this anomaly might quite well be due to contamination. Recrystallisation gave fraction 3a, and though there was not enough material to allow a redetermination of the mol. wt. it will be seen that there is good agreement for the rest of the data with the mixture 85% C₂₃ + 15% C₂₅. We believe therefore that fraction 3 is a mixture 83% C₂₃ + 17% C₂₅ with a trace of C₂₁.

Table III. *Melting points and long crystal spacings of mixtures of n-docosanoic and n-tetracosanoic acids.*

Percentage composition		M.P. (°C.)	Spacings in Å.		
C ₂₃	C ₂₄		Series no.	Pressed layer B	Melted layer C
100	0	80-80.2	—	53.5	48.3
90	10	78.2-78.4	B 422	54.4, 50.2	49.2
80	20	74.9-75	B 423	53.5	49.17
70	30	74.5-74.6	B 420	53.3	53.8
60	40	75.4-75.5	B 424	56.5	53.6
50	50	75.5-75.7	B 421	56.3	54.5
0	100	84-84.2	—	57.7	52.6

The spacing data for fraction 5 limit the choice of the two comparison mixtures to 85 % C_{23} + 15 % C_{25} and the fit throughout is good.

It will be noticed that certain odd-even chain mixtures in Table IV have spacings which agree well with those found for the fractions discussed above, but all such mixtures are definitely ruled out by their m.p. and mol. wt.

Reduction of phrenosinic acid. We have repeated the experiments of Klenk [1928, 2], but with only 0.37 g. of phrenosinic acid available the results were inconclusive. Much neutral material was obtained and the acidic products (0.14 g.) on fractionation gave a small amount of acid, m.p. 78.0–78.5°, which, when viewed under the microscope, was seen to consist of masses of broken leaflets having no recognisable cleavage angles or edges. As one would expect, the X-ray photograph was too poor for interpretation.

The constitution of phrenosinic acid. Reviewing briefly the evidence given above we are able to state that the phrenosinic acid obtained from the sample of phrenosin prepared by Dr Rosenheim was definitely a mixture of even-number α -hydroxy-acids of mean mol. wt. corresponding closely to C_{24} , which on oxidation gave a mixture of odd-number n -fatty acids, the main bulk of which was definitely identified as 85 % C_{23} + 15 % C_{25} . We feel justified therefore in drawing the conclusion that the phrenosinic acid mixture consisted in very large part of α -hydroxy- n -tetracosanoic acid (85 %) and α -hydroxy- n -hexacosanoic acid (15 %). Our findings are thus in substantial agreement with those of Klenk, and we have no doubt that by fractional distillation of the methyl esters of the oxidised acids he obtained n -tricosanoic acid in good yield. We differ from him only on the composite nature of the phrenosinic acid, a point which we feel needs further discussion, because the components will vary somewhat according to the degree of purification to which the parent phrenosin has been subjected, and therein we believe lies the origin of the discrepancies between his results and Levene's.

Our own results leave no doubt that phrenosin, like lecithin, kephalin and (as we show later) kersin, is a mixture, the components of which differ only in the chain length of the fatty acid. As Dr Rosenheim's sample of phrenosin was exhaustively freed from kersin and then recrystallised 4 times from pyridine, it would naturally represent a phrenosin rich in the more insoluble (i.e. longer-chain acid) components. Levene and Taylor's earlier sample had also been rigidly purified, and the acids obtained on oxidation of the phrenosinic acid had a slightly higher mol. wt. (367) than ours. But their later sample, which they required in large bulk, was clearly not so well purified, for the oxidised acids in this case had a definitely lower mol. wt., and in our opinion show clearly the presence of n -heneicosanoic as well as n -tricosanoic and n -pentacosanoic acids. The lower-boiling fraction, m.p. 74–75°, mol. wt. 341, which they suggested was docosanoic acid, is clearly about 40 % C_{21} + 60 % C_{23} (m.p. 73.5°, mol. wt. 343) and their higher-melting fraction, m.p. 79.5–80°, mol. wt. 367, which they suggested was "lignoceric" acid, is clearly about 40 % C_{23} + 60 % C_{25} (m.p. 77.8°, mol. wt. 371) in both instances allowance being made for the difference between Levene's and our own methods of taking melting-points. There is no doubt therefore that brain contains at least three phrenosins, whose acidic components are α -hydroxy- n -docosanoic, α -hydroxy- n -tetracosanoic and α -hydroxy- n -hexacosanoic acids respectively and that following Klenk's [1928, 2] suggestion, they are the α -oxidation products of the corresponding kersins.

2. *Thudichum's neurostearic acid.*

Through the kindness of Dr Rosenheim and Sir Henry Dale we have been able to examine a sample of Thudichum's original preparation of neurostearic acid, which is now preserved together with many other of his preparations at the National Institute for Medical Research [cf. Rosenheim, 1930]. The label on the specimen bottle bore the inscription "Neurostearic Acid, m.p. 85.5°; C=75.88%, H=12.85%, O=11.27%", showing that it was the identical specimen described by Thudichum [1879].

We found that the mol. wt. by titration, which Thudichum did not determine, was 381 and that for a 6.8% solution in pyridine the observed rotation was $\alpha_D^{18} + 0.06^\circ$, whence $[\alpha]_D^{18} = +0.9^\circ$. These values suggest that the preparation contained approximately 25–30% of phrenosinic acid, the remainder being "lignoceric" acid. 0.5 g. in 10 ml. of boiling methyl alcohol was treated with 1 ml. of 10% lithium acetate in the same solvent and the precipitated soap filtered hot (0.25 g.). This was suspended in 20 ml. of methyl alcohol, which was then boiled under reflux for 7 hours. The insoluble residue was filtered hot (0.12 g.) and the free acid recovered (0.11 g. m.p. 89–90°). The treatment with alcoholic lithium acetate (0.25 ml.) was then repeated, giving 60 mg. of fairly pure phrenosinic acid, m.p. 97.0–97.5°. (Found: C, 75.6; H, 12.7%; mol. wt. by titration 394.) For a 2.4% solution in pyridine the observed rotation was $\alpha_D^{18} + 0.1^\circ$, whence $[\alpha]_D^{18} = +3.7^\circ$.

3. "*Lignoceric*" acid from kersin.

The acid generally referred to as "lignoceric" acid, which has mol. wt. corresponding to $C_{24}H_{48}O_2$ and m.p. constant at 80–81° under the usual conditions of purification, is the constituent fatty acid of kersin and sphingomyelin and can be isolated from many naturally occurring fats and waxes. Meyer *et al.* [1913] were the first to show that it was not identical with synthetic *n*-tetracosanoic acid, which melted at 84.5°, and it became customary to regard the lower-melting acids as an *isotetracosanoic* acid with a branched chain. Brigl and Fuchs [1922] however examined the acid (m.p. 80–81°) prepared from beechwood tar, and isolated from it *n*-tetracosanoic acid, m.p. 84.5°, identical with the synthetic acid, and an isomeride, m.p. 74.5°, which they called *isolignoceric* acid. Levene *et al.* [1924], applying Brigl and Fuchs's methods to the lignoceric acid from arachis (peanut) oil and from kersin, were unable to isolate any acid melting higher than 80.5–81° and reiterated the former view that lignoceric acid was not identical with *n*-tetracosanoic acid. Klenk [1927] however, who obtained the acid, m.p. 80–81°, from kersin and fractionated it with methyl alcoholic lithium acetate, isolated a product, m.p. 83–84°, which was identified as *n*-tetracosanoic acid. More recently Jantzen and Tiedcke [1930] have fractionated the higher-melting fatty acids from arachis (peanut) oil in a new type of still and have obtained *n*-eicosanoic acid, *n*-docosanoic *n*-tetracosanoic acid (in largest amount) together with a residue of longer-chain acids. The purity of the fractions was checked against synthetic products, and no evidence for the presence of "*isobehenic*" or "*lignoceric*" acid was obtained. Further, the crystal spacings of the two acids of Brigl and Fuchs have been examined [Francis *et al.* 1930]. That melting at 84.5° is definitely *n*-tetracosanoic acid, whilst the *isolignoceric* acid, m.p. 74.5°, showed no evidence of possessing a branched chain and had spacings of 57.2 Å. when pressed and of 52.52 Å. when melted, values which are similar to those of 50% C_{23} + 50% C_{24} , a mixture which melts at 79.8° and has spacings of 57.5 Å. and 52.0 Å. It was suggested on this evidence that C_{23} and C_{24} were probably the main components of this mixture, but recent experience has led us

to believe that mixtures of odd- and even-number acids do not occur in nature. It is probable that the isolignoceric acid was not a binary mixture but a more complicated mixture of the even-number acids, C_{22} , C_{24} , C_{26} , though we have not sufficient comparative data to suggest a percentage composition. Taken as a whole the composite nature of "lignoceric" acid appears to us to be definitely proven and in the present research we have sought to do no more than suggest the possible composition of the acid from kersin.

4.0 g. of kersin, of the same degree of purity as that described by Rosenheim [1914; 1916], when treated according to his directions gave 1.35 g. of "lignoceric" acid. After several crystallisations from acetone at room temperature this melted at $80.3-80.5^\circ$. (Found: C, 78.4, H, 13.1%, mol. wt. by titration 369. $C_{24}H_{48}O_2$ requires C, 78.2, H, 13.1%, mol. wt. 368.4.) These values are in general agreement with those of other workers. The crystal spacings (B 446) were 59.1 \AA . pressed and 52.45 \AA . melted, the plate having 7 sharp orders with the second well developed. The acid is definitely not a mixture of odd-number acids, and if a binary number of even-number acids, the m.p. and spacings suggest approximately 20% C_{22} + 80% C_{24} . But the observed mol. wt. is too high for such a mixture and, being equal to that of the C_{24} acid, suggests a ternary mixture in which this acid is accompanied by small and approximately equal amounts of C_{22} and C_{26} . We accordingly prepared two such mixtures (1) 10% C_{22} + 80% C_{24} + 10% C_{26} . The m.p. was $80.8-81^\circ$ and the crystal spacings were (B 431) 59.1 \AA . pressed and 53.2 \AA . melted, the plate being comparable in all respects with that of the "lignoceric" acid (B 446). (2) 15% C_{22} + 70% C_{24} + 15% C_{26} . The m.p. was $79.1-79.3^\circ$ and the X-ray photograph (B 432) showed only 5 diffuse orders giving a C spacing in both the pressed (55.7 \AA .) and melted (54.8 \AA .) layers. The mixed m.p. of mixture (1) with the "lignoceric" acid was $80.5-80.7^\circ$, and in our opinion the former represents very closely the composition of the latter. At least three kerasins therefore are present in brain, containing *n*-docosanoic, *n*-tetracosanoic and *n*-hexacosanoic acids as their acidic components respectively, and although we have no direct evidence on the point we feel that the same conclusion applies to sphingomyelin, which also contains "lignoceric" acid, m.p. $80-81^\circ$.

Many different mixtures of the *n*-acids, C_{20} , C_{22} , C_{24} , C_{26} and perhaps C_{28} can have m.p. $80-81^\circ$ and mol. wt. about 368 and such undoubtedly occur widespread in nature and have hitherto all been referred to as "lignoceric" acid. But the pure C_{24} acid, m.p. 84.5° , has always been referred to by former workers as *n*-tetracosanoic acid, and it seems to us that the continued use of the name "lignoceric" acid for the product m.p. $80-81^\circ$ tends to obscure its composite and varying nature with the result that unjustifiable conclusions as to its metabolism, based on the assumption that its molecule contains 24 (6×4) carbon atoms are still being drawn [cf. Klenk, 1928, 2]. We therefore recommend that the name "lignoceric" acid be abandoned, and that henceforth such acids be referred to simply as "mixed fatty acids m.p. $80-81^\circ$ " [cf. "melissic" acid, *etc.*: Chibnall *et al.* 1934]. As long-chain α -hydroxy-acids occur, according to our present knowledge, only in cerebrosides, there seems less objection to the retention of the name "phrenosinic" acid.

4. Melting points and long crystal spacings of certain mixtures of odd- and even-number *n*-fatty acids.

The following series of mixtures of *n*-fatty acids have been prepared and examined during the course of the present research, and are to be regarded as an extension of the work of Piper *et al.* [1934]; the X-ray data include the spacings

observed for both pressed and melted layers. The series covers all possible binary combinations of the acids C_{21} – C_{25} , and the M.P. data, which call for no special comment, are given in Tables III and IV and graphically in Fig. 1.

Long crystal spacings of mixtures of n-docosanoic and n-tetracosanoic acids. Measurements of a series of mixed even-number *n*-fatty acids, C_{28} and C_{30} , have been given [Piper *et al.* 1931] and we now give some further measurements of a few mixtures of C_{22} and C_{24} acids. The latter are slightly more comprehensive in that they show the spacings in both B and C forms throughout the range of proportions investigated.

*The crystal forms and transitions for pure odd-number *n*-acids and their mixtures.* The odd-number acids appear in four modifications A, B, C, D [de Boer, 1927]. The A form has a vertical chain and has not been observed in pure acids containing more than 17 carbon atoms; B, C and D are characterised by increasing tilts of the chains. For chain lengths of 19 or more carbon atoms the B form is stable and transforms reversibly to C at a temperature a few degrees below the M.P. The B form is therefore usually adopted by crystals obtained from solution or from the melt, though sometimes the C form is stabilised by rapid cooling or by the presence of small amounts (up to 15%) of a homologous impurity. We have not observed the D form for chain lengths greater than 15 carbon atoms.

The binary mixtures of odd-number acids give results which are numerically consistent. The spacings depend much less on the treatment of the specimen than is the case for the mixtures of even-number acids, and do allow one to fix the proportions of a binary mixture with a reasonable degree of certainty. The measurements are plotted in Fig. 2 and allow some straightforward generalisations.

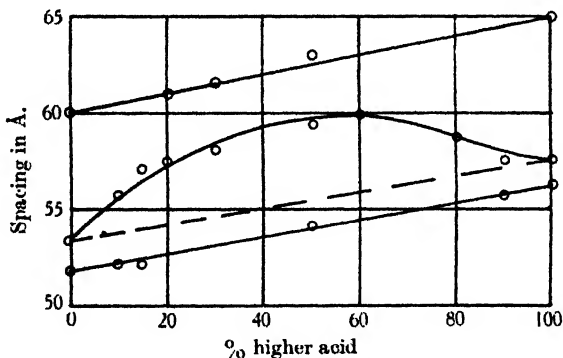


Fig. 2. Crystal spacings, $C_{23} + C_{25}$ acids.

(1) When A and C spacings appear they lie in two straight lines joining the spacings of the pure acids. This is what would be expected on any simple theory, but has not been observed in any other mixtures of aliphatic compounds with which we have made measurements.

(2) The B spacings lie on a smooth curve which rises well above the line joining the spacings of the pure acids. Thus, when both B and C spacings occur there is in general a much greater numerical difference between them than for mixtures of two even-number acids. A B spacing lying on this curve has with a single exception been observed for every pair of odd-number acids examined.

(3) For proportions of 0 to 15% of the longer-chain acid we obtained a B spacing from a pressed and C from a melted layer, but no A spacing.

Table IV. *Melting-points and long crystal spacings of certain odd-number and odd-even-number binary mixtures of n-fatty acids.*

Percentage composition		M.P. °C.	Series no.	Crystal spacings in Å.			
C ₂₃	C ₂₅			Pressed layer (P) or melted layer (M)	A	B	C
100	0	79.4-79.6	—	—	60*	83.5	51.8*
90	10	77.8-78.0	B 412	{ P M	— —	55.7 —	— 52.1
85	15	76.9-77.1	B 438	{ P M	— —	57.1 —	— 52.1
80	20	76.1-76.3	B 413	{ P M	60.95 —	57.45 57.1	— —
70	30	76.0-76.3	B 414	{ P M	61.6 —	58.1 58.4	— —
50	50	77.0-77.2	B 415	{ P M	63 —	59.4 —	— 54.1
40	60	77.7-77.8	B 440	{ P M	— —	59.9 59.2	— —
30	70	78.7-78.8	B 439	{ P M	— —	59.5 59.6	— —
20	80	80.2-80.4	B 434	{ P M	— —	58.7 59.3	— —
10	90	81.8-82.0	B 433	{ P M	— —	57.5 —	— 55.6
0	100	83.2-83.4	—	—	65*	57.8	56.2*
C ₂₅	C ₂₇						
50	50	80.9-81.1	B 398	P	67	64.25	—
70	30	80.5-80.7	B 397	P	66.34	—	—
C ₂₁	C ₂₃						
10	90	77.7-78.0	B 430	{ P M	— —	53.25 —	— 51.65
C ₂₃	C ₂₄						
90	10	79.0-79.2	B 417	{ P M	— —	57.1 57.0	54 51.8
80	20	78.8-79.0	B 416	{ P M	62.3 —	56.6 57.2	— 52.4
20	80	82.0-82.2	B 437	{ P M	— —	58.0 —	52.8 —
10	90	83.0-83.2	B 436	{ P M	— —	57.65 —	52.4 52.6
C ₂₂	C ₂₃						
90	10	78.6-78.8	B 425	{ P M	— —	52.8 —	48.1 49.0
80	20	77.6-77.8	B 426	{ P M	— —	54.0 —	49.0 —
70	30	76.4-76.6	—	P	—	53.3	—
20	80	77.5-77.7	B 428	{ P M	— —	53.3 —	— 51.25
10	90	78.1-78.3	B 427	P	—	52.5	—

* Extrapolated.

(4) For proportions of 20 % to 50 % of the longer-chain acid both A and B spacings appeared for the pressed and a B spacing for the melted layer. The equimolar mixture was an exception and had a C spacing for the melted layer.

(5) Proportions of 60 % to 80 % of the longer-chain acid gave only a B spacing for both pressed and melted layers.

(6) 90 % or more of the longer-chain acid gives results similar to those for the 0-15 % mixtures.

The measurements are recorded in Table IV and are shown graphically for the mixtures of *n*-tricosanoic and *n*-pentacosanoic acids in Fig. 2.

Additional assistance in distinguishing between odd- and even-numbered chains of the series of *n*-acids is given by a slight but perceptible difference in the intensity distribution, for the even orders are definitely weaker in the odd-number series. We have found that if the ninth order of reflection is visible then if the mixture is of even-number acids, the second order will appear, but if the mixture is of odd-number acids there is frequently no observable trace of any even order. But the intensity distribution in mixtures is variable, and evidence of this nature is suggestive and corroboratory rather than definitive.

Photographs have been taken of several odd-even binary mixtures, and the spacings are recorded in Table IV. The behaviour of such mixtures is complicated and has not been reduced to a set of rules, but where we have obtained spacings agreeing numerically with those of mixtures from natural sources we have always found that there is no accord in the m.p. and mol. wt. measurements. Since the behaviour of the oxidised acids from phrenosinic acid strongly suggests that they are mainly composed of two acids, we consider that there is no evidence to support a suggestion of a mixture of odd- and even-number acids.

SUMMARY.

Brain contains at least three phrenosins whose acidic components are α -hydroxy-*n*-docosanoic, α -hydroxy-*n*-tetracosanoic and α -hydroxy-*n*-hexacosanoic acids respectively. Phrenosinic (cerebronic) acid is a mixture of these acids. Brain also contains at least three kerasins, whose acidic components are *n*-docosanoic, *n*-tetracosanoic and *n*-hexacosanoic acids respectively. Evidence that "lignoceric" acid is a mixture is summarised and the suggestion made that the name is misleading and should be abandoned.

Several binary mixtures of *n*-fatty acids of chain length from C_{21} to C_{25} have been prepared, and their melting points and crystal spacings are recorded.

In conclusion we should like to record our grateful thanks to Dr Rosenheim for providing us with samples of highly purified brain cerebroside and for the interest he has taken in this work. Our thanks are also due to Prof. Francis and Dr Malkin for kindly providing us with samples of pure synthetic higher *n*-fatty acids.

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XIX. THE DIETARY PREVENTION OF FATTY LIVERS. TRIETHYL- β -HYDROXYETHYL-AMMONIUM HYDROXIDE.

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THE discovery of the action of choline in preventing fat accumulation in the livers of rats [Best and Huntsman, 1932] has been considerably extended, and it has become apparent that in a variety of experimental conditions this substance exercises both preventive and curative actions on fat deposition in the liver [Best and Huntsman, 1935]. The fact that choline is the base present in lecithin makes it possible that its action may be exercised through synthesis of that substance, in accordance with the original suggestion of Leathes that fatty acids might be transported from the liver to the tissues after incorporation in the phosphatide molecule. Light should be thrown on this question if a base could be found which possessed an action on liver fat similar to that of choline and which could be shown to be present in the liver and other tissues in very small amount by isolation of a suitably characteristic derivative. Such a base might be found to have been incorporated in a new phosphatide molecule in which it had replaced the choline of lecithin. Its absence from the phosphatide would not exclude the possibility that choline exercised its action by enabling lecithin synthesis to occur, but would perhaps make that mode of action less likely. Its presence however would provide a means for investigation of the whole problem from many new aspects.

We have therefore begun a study of the action of substances chemically similar to choline. Whilst considerable difficulties have been encountered because of the toxicity of these compounds, we are able to report in the present paper that triethyl- β -hydroxyethylammonium hydroxide, which differs from choline only in the replacement of the three methyl groups of that substance by ethyl groups, possesses an action on liver fat similar to that of choline. It will be recalled that in their original work Best and Huntsman [1932] reported that betaine was effective, whilst aminoethyl alcohol was without action. The former finding might indicate an action independent of lecithin, whilst the latter seems to preclude kephalin from being involved.

EXPERIMENTAL.

In each experiment three groups of rats were used and all received the basal fatty liver-producing diet, which consisted of beef dripping 40, glucose 45, extracted caseinogen 5 and salt mixture 5, while marmite, 5 parts, was used as a source of B vitamins and vitamins A and D were supplied as cod-liver oil (1 drop per animal every 3 days). One group served as control, whilst in addition to the basal diet the second received triethyl- β -hydroxyethylammonium hydroxide and the third choline, in order to obtain some quantitative comparison of the action with that of choline. These groups are designated "control",

"derivative" and "choline" in the table and discussion which follow. The choline content of the basal diet was about 1.7 mg./100 g. The bases were used as their chlorides and were administered by the methods set out in the table. The animals were kept in groups of two to four per cage and their average daily food intake was determined.

Preparation of triethyl- β -hydroxyethylammonium hydroxide.

Molecular proportions of triethylamine and ethylenechlorohydrin were heated in a sealed tube at 95° for 4 hours. To a solution of the resulting product in absolute alcohol were added acetone and ether. The exceedingly deliquescent fine crystalline material was redissolved in alcohol and similarly reprecipitated four times. (Found: Cl 19.8, N 7.5; $C_8H_{20}ONCl$ requires Cl 19.55, N 7.7 %.) The chloroaurate melted at 225° (C 19.7, H 4.8, N 3.12, Au 40.5 %; $C_8H_{21}ONAuCl_4$ requires C 19.7, H 4.3, N 2.88, Au 40.6 %).

The results of Exps. 1 and 2, in which the livers of all the groups were treated as pooled samples, show that the derivative has prevented the accumulation of liver fat, Exp. 2 suggesting that its action is at least equivalent to that of choline in the amounts given. In both experiments, however, the food consumption of the derivative group was substantially less than that of the control animals, whilst the weight losses were considerably greater. Although weight losses of this magnitude do not affect the percentage of fat in the liver [Best *et al.* 1935], at the same time the much lower food intake in Exp. 2 and the greater toxicity of the compound under examination made further experiments desirable in order to attempt to overcome the possible effects of these factors on the results. In all the remaining experiments the individual livers were investigated. Exps. 3 and 4 were then carried out by administration of a solution of the substance orally by drops and by stomach tube respectively, on the assumption that the exceedingly bitter taste of the derivative was rendering the diet unpalatable. The control and choline groups in these two experiments received solutions of sodium chloride and of choline chloride respectively, administered by the same methods in order to avoid any interfering effect of the administration techniques.

The effect of the compound was shown by the result of Exp. 3, in which the food intake of the derivative group compared with that of the control group was better than in Exp. 2, with closely similar body weight changes, although the effect seemed less than that of choline at an approximately similar daily dosage in contrast to Exp. 2. This may be connected with the fact that the method of administration caused so intense a salivary flow that much of the derivative may have been lost in this way. A further fact was that whereas the individual values for the does ranged from 5.7 to 8.8 % (mean 7.4 %), for the derivative group, compared with the control range of 8.7–24.0 % (mean 14.75 %)—a satisfactory result—for the bucks of the derivative group the range 6.6–11.8 % showed a considerable overlap with the control bucks, 8.4–31.0 %. Evidence regarding this overlap was obtained in further experiments.

The result of Exp. 4 was again satisfactory, for the livers of the derivative bucks contained 9.2 % "fat", against the control value of 33.1 % and the choline group value of 8.5 %, although the food intake was somewhat less than that of the controls. Thus 27.8 mg. derivative seemed just as effective as 26.9 mg. choline. In this Exp. there was no overlap in the extreme values (controls 19.6–42.4 %; derivative group 4.8–12.0 %).

The results of Exp. 4 led us in Exp. 5 to attempt to observe the effects further by incorporation of the substance in the diet as in Exps. 1 and 2, in the hope that

Table I. *The effect of triethyl-β-hydroxyethylammonium hydroxide in preventing fat deposition in the liver.*

No. of exp.	Duration of exp. (days)	Method of administration of choline or derivative	Choline or derivative ingested/animal/day (mg.)	Group	No. and sex of animals	Wt. gain or loss (%)	Diet eaten per animal per day (g.)	Liver wt. % body wt. (g.)	Fatty acids and unsp. matter in liver (%)	Extreme limits of figures averaged in preceding column	Wt. of fatty acids and unsp. matter in liver of 100 g. rat (g.)
1	17	Incorporated as an aqueous solution in diet	*	Control	6 M & F	- 8.4	9.6	4.1	24.1	Pooled sample	0.99
				Choline	6 "	- 7.7	9.3	3.1	5.2		0.16
				Derivative	6 "	- 10.6	7.3	3.3	7.1		0.23
2	20	As in 1	—	Control 1	3 F	- 7.6	10.0	3.3	20.7	Samples pooled	0.69
			—	" 2	3 F	- 3.7	10.0	4.1	30.5	according to body weight changes	1.25
			—	" 3	3 F	+ 4.3	10.0	3.3	20.0		0.66
			79.8	Choline 1	5 M	+ 3.0	9.2	2.8	8.9		0.25
				" 2	5 M	- 2.2	9.2	2.8	8.1		0.23
			47.6	Derivative	10 M & F	- 13.6	5.3	3.1	7.0		0.22
3	18	Aqueous solution orally from buccette	—	Control 1†	4 F	- 4.2	7.4	4.4	14.75	8.7-24.0	0.65
			—	" 2	5 M	- 3.0	8.9	3.6	10.75	8.4-31.0	0.60
			61.6	Choline 1	5 F	+ 1.2	7.9	3.8	5.2	4.1- 6.9	0.20
				" 2	5 M	+ 1.0	9.5	2.8	5.3	4.5- 6.1	0.15
			67.4	Derivative 1	5 F	- 7.7	5.9	3.8	7.4	5.7- 8.8	0.28
				" 2	5 M	- 6.1	8.1	3.7	9.6	6.6-11.8	0.36
4	18	As aqueous solution by stomach tube	—	Control 1	6 F	- 4.7	8.8	4.1	19.1	8.3-32.6	0.78
			—	" 2	6 M	- 8.8	8.8	5.8	33.1	19.6-42.4	1.92
			26.9	Choline 1	6 F	+ 1.6	9.1	3.1	5.9	4.8- 6.9	0.18
				" 2	6 M	- 2.9	9.1	3.3	8.5	6.2-13.7	0.28
			27.8	Derivative 1	1 F†	- 9.4	7.0	3.7	5.1	—	0.19
				" 2	6 M	- 13.4	7.0	4.0	9.2	4.8-12.0	0.37
5	19	As in 1	—	Control 1	5 F	- 8.7	8.4	4.7	26.1	14.5-38.8	1.23
			—	" 2	5 M	- 6.9	8.4	4.6	30.3	17.4-39.2	1.39
			20.8	Choline 1	5 F	+ 3.3	9.5	3.2	5.8	4.6- 7.3	0.19
				" 2	5 M	+ 0.5	9.5	3.3	7.5	6.3- 9.8	0.25
			27.8	Derivative 1	5 F	- 1.9	8.8	3.4	8.8	5.9-12.5	0.30
				" 2	5 M	- 7.5	8.8	3.9	18.7	12.7-29.4	0.73
6	13	As in 1	—	Control 1	8 F	- 0.2	8.0	4.7	19.6	12.8-36.7	0.92
			—	" 2	8 M	- 5.8	8.0	6.4	31.4	15.2-39.9	2.01
			8.8	Choline 1	8 F	+ 2.9	8.4	3.8	8.3	6.3-10.2	0.32
				" 2	8 M	- 2.4	8.4	4.2	8.1	6.8- 9.8	0.34
			18.3	Derivative 1	8 F	- 5.5	7.0	3.7	8.0	5.6-10.3	0.30
				" 2	8 M	- 3.8	7.0	4.8	18.7	8.9-27.8	0.90

* Actual amounts of choline and derivative not measured. The diets contained however 10 mg. of each per g. diet.

† One animal died. ‡ Five animals died.

the low amount of the substance used would render the diet more palatable. In this experiment equimolar concentrations of choline and the derivative were used, although the better food consumption of the choline group rendered the actual intakes of choline and the derivative slightly different. The food intake and body weight changes were strictly comparable in the control and derivative groups and the results of more certain value. These were similar to those of Exp. 3, for whereas the results with the does were entirely satisfactory, the bucks again showed apparently less reaction to the derivative (\bar{x} control 17.4–39.2 %, mean 30.3 %; derivative 12.7–29.4 %, mean 18.7 %; choline 6.3–9.8 %, mean 7.5 %).

Exp. 6 was carried out by the same method, but at still lower concentrations of choline and the derivative. Here again the results for the does were completely positive (control 12.8–36.7 %, mean 19.6 %; derivative 5.6–10.3 %, mean 8.0 %), whilst those of the bucks were very similar to those of Exp. 5 (control 15.2–39.9 %, mean 31.4 %; derivative 8.9–27.8 %, mean 18.7 %).

The results of Exps. 1–6 showed therefore that the compound adequately prevented the deposition of fat in the livers of the does and that in this action it was almost as potent as choline. Its effect seemed far less pronounced however with the bucks, as was shown in all four Exps. (3–6) in which individual values were investigated. No obvious explanation appeared for this. The wide range of variation of the liver fat in the control groups of animals, which always occurs, makes any result of this kind liable to suspicion and accordingly a more elaborate experiment was carried out, in which individual food intakes were measured and every effort made to substantiate the findings by adopting as rigorous a method as possible.

In this experiment 72 animals were used and divided into three groups as before, each rat being kept in a separate cage. Of the 24 rats in each group, 12 were does and 12 bucks and of these 6 does and 6 bucks were chosen so that they were from the same litters as the corresponding animals in the other two groups. Thus for each rat in the derivative group there were corresponding litter-mates in the other two groups. The remaining 12 animals in each of the 3 groups (6 bucks and 6 does) were taken from stock and thus served for comparison with the 12 litter-mates also in each group. Further, in order to control as far as possible any effect of varying food intake, the 24 animals of the derivative group, in which in Exps. 1–6 the food intake had tended to be lower, were placed on their diet a day before the choline and control groups. Then throughout the experiment the animals of the latter two groups were fed as nearly as possible with the same amount of food as the derivative animals had consumed during the previous day. In this way at the close of the experiment, which lasted 13 days, the food intake of rat No. 1, say, in the control group was almost the same as that of each of the rats No. 1 in the other two groups and so on.

The results of the experiment are represented diagrammatically in Fig. 1, in which the values for the control animals were plotted in ascending order of magnitude, the lowest being that of rat No. 10, and the highest that of No. 6. The figures for the corresponding animals in the other two groups were then placed in position and the curves obtained as shown. There also appears on the diagram the food intake for the rats of the control group which, as already stated, was almost identical with that of the corresponding members of the other two groups because of the method of feeding adopted.

Certain conclusions may readily be drawn from the figure. In the first place, although the food intake varies from 6.3 to 9.8 g. and the fat content of the liver in the case of the controls from 12.7 to 35.8 %, yet there is no relationship

whatever between them and this is equally true for the other two groups. The individual variation cannot therefore be explained by differences in food intake. In the second place the large individual variation is in no way dependent on whether the rats were litter-mates or simply selected in the usual way from stock. This is clear when it is considered that Nos. 1-12 in any group on the diagram were litter-mates of the corresponding numbers in the other two groups, the variation being as great amongst Nos. 1-12 as amongst Nos. 13-24.

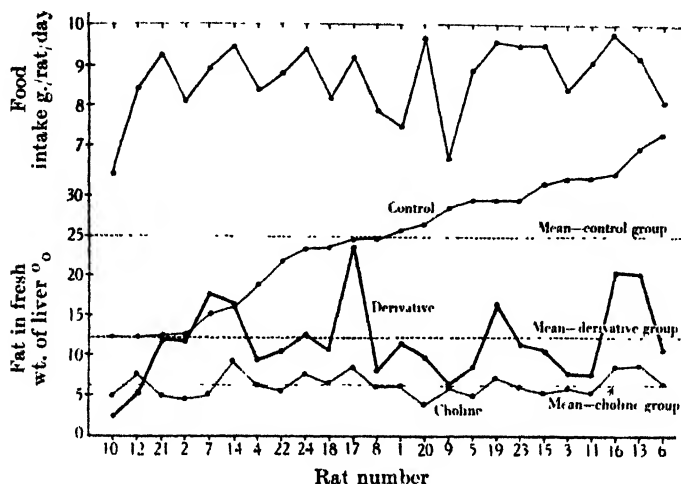


Fig. 1. Percentage of fat in liver of rats with their food intake. The average amount of choline and of derivative eaten per animal per day was 18.2 and 22.4 mg. respectively.

Furthermore from the lines showing the mean values in the three cases it is obvious that, on the average and with the amounts of each substance ingested, the derivative has had an action equal to about 2/3 that of choline, the actual figures being 24.8 (control), 6.4 (choline) and 12.2 % (derivative), a reduction of 18.4 units % with choline and of 12.6 units % with the derivative. Although this is true for the average of all 24 animals in each group, it must again be pointed out, as in previous experiments, that the average figure for the does (control 23.7, choline 5.9 and derivative 9.5 %) was again smaller and the derivative apparently more effective than in the case of the bucks (25.9, 6.9 and 14.6 % respectively). This also appears from the diagram to be independent of the amount of food ingested.

Therefore although the problem of the large individual variation so often encountered in these and in similar experiments remains unsolved, the action of the derivative has again been confirmed.

One further point of interest is that the figure shows that the curve of the liver fat of the choline animals follows in a general way that of the corresponding derivative animals in that it tends to rise and fall at the same time, although in a far more limited degree.

A final experiment was now performed to ensure that there was no abnormal excretion of fat in the faeces of the rats receiving the derivative. Two groups of six animals, each containing three bucks and three does, were fed for 15 days on the 40 % fat diet, one group receiving the diet only, the other being given the derivative in addition. The results showed that both groups of animals excreted 0.22 g. faecal fatty acids per rat per day.

In view of the findings that the derivative exercises an effect in so small a daily dose, it is proposed to repeat some earlier experiments which were abandoned at that time on account of the toxicity of the products. In these experiments trimethylamine, trimethyl- γ -hydroxypropylammonium hydroxide and tripropyl- β -hydroxyethylammonium hydroxide were used in the hope that these alterations might throw light on the nature of the active group. Further experiments are also in progress with the derivative used in the present work, with a view to attempting to utilise the preventive action of this compound on liver fat as a means of investigating its mode of action by the methods indicated earlier in this paper.

SUMMARY.

1. The preventive effect of triethyl- β -hydroxyethylammonium hydroxide in controlling the percentage of fat in the livers of rats was studied in seven separate experiments.

2. This compound was shown to have a preventive action, although the effectiveness of its action was less than that of choline.

3. Furthermore it was found to have a greater action in the control of liver fat in the case of does than it had in the case of bucks.

4. The large individual variations found in rats of the same groups remains unexplained. Attempts to control these variations by use of litter-mates for the different groups and of controlled food consumption did not throw light on the variations, which appear to be independent of food intake in the conditions of these experiments.

We wish to express our thanks to the Medical Research Council for a grant from which the expenses of this research were defrayed.

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XX. THE EFFECT OF LIVER FEEDING ON THE "FAT" CONTENT OF THE LIVER.

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THE investigations recorded in this paper were carried out as a result of the findings of Blatherwick *et al.* [1931; 1932; 1933, 1, 2] on the effect on the "fat" content of the liver of the feeding to rats of diets consisting largely of fresh liver, dried liver or certain liver extracts. These authors observed that feeding raw or dried liver resulted in the production of fatty livers; that the addition of lecithin to the diets had no effect in preventing the fatty liver; and further that it appeared possible that there was present in the liver a water-soluble substance, which was responsible for the deposition of liver "fat", or alternatively that aqueous extraction of liver caused some change in the constituents of the liver residue, as a result of which the amply demonstrated property of cholesterol in causing "fat" deposition in the liver was opposed [Okey, 1933, 1, 2; Chanutin and Ludewig, 1933; Best *et al.*, 1934; Channon and Wilkinson, 1934].

Consideration of the relative amounts of fat and cholesterol in liver led us to think that the apparent failure of lecithin to influence the fat deposition was largely concerned with the high proportion of fat and cholesterol present in such diets. The provision of 2.3% choline in a synthetic diet containing 2% of cholesterol and 20% of fat will not entirely prevent cholesteryl ester deposition [Best *et al.*, 1934]. Hence it might be anticipated that the addition of further lecithin to a liver diet, which would already contain some 13% phosphatide as well as 1% cholesterol and 30% fat, might not cause any further decrease in "fat" deposition, since the maximum choline effect was already being exercised by the lecithin contained in the liver.

In order to investigate this possibility, the work described in this paper on the feeding of liver to rats was carried out. The effect of the withdrawal from the dietary liver of the constituent lecithin, cholesterol and glyceride, and the replacement of these substances by an equivalent amount of cholesterol or beef dripping, either singly or together, has been studied. In other experiments more cholesterol than was originally present in the liver has been added. By these means a series of results of a strictly comparable nature has been obtained and from them the effect of the individual constituents of dietary liver in fatty liver production may be assessed.

In the work of Blatherwick *et al.* [1933, 2] the results of the different experiments are not strictly comparable, because each diet did not contain the equivalent of the constituents of the original liver and in some cases the extracts employed were not from the main bulk supply. At the same time opportunity was taken to investigate the possibility of the presence in liver of a water-soluble factor responsible for fatty liver production.

EXPERIMENTAL.

Composition of the diets.

The composition of the diets is set out in Table I.

Table I. *Composition of the diets.*

Diet No.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Raw liver	—	—	—	—	—	—	—	—	100	100	100
Dried liver	75	75	—	—	—	—	—	100	—	—	—
Dried liver after alcoholic extraction	—	—	—	51.8	51.8	—	—	—	—	—	—
Dried liver after aqueous extraction	—	—	52.8	—	—	—	—	—	—	—	—
Dried liver after alcoholic and aqueous extraction	—	—	—	—	—	29.6	29.6	—	—	—	—
Residue after evaporating aqueous extract of dried liver	—	—	—	—	—	—	22.2	—	—	—	—
Marmite	5	5	5	5	5	5	5	—	—	—	—
Salt mixture	2	2	2	2	2	2	2	—	—	—	—
Glucose	17	15	39.2	17	17	37.2	15.0	—	—	—	—
Cod-liver oil	1	1	1	1	1	1	1	—	—	—	—
Choline chloride	—	2	—	—	—	—	—	—	—	0.2	0.4
Beef dripping	—	—	—	23.2	22.45	22.45	22.45	—	—	—	—
Cholesterol (as present in liver)	—	—	—	—	0.75	0.75	0.75	—	—	—	—
Cholesterol	—	—	—	—	—	2.0	2.0	—	—	—	—

The liver and preparations made from it were obtained as follows.

Dried liver. This was a powdered preparation which had been specially prepared through the kindness of Dr F. H. Carr of the British Drug Houses, Ltd. by drying liver *in vacuo* below 60°. Analysis showed that this preparation contained 30.91% of ether-alcohol-soluble material and 29.5% of water-soluble material; the ether-alcohol-soluble material was completely analysed, and the original dried liver was found to contain 13.4% "lecithin", 0.32% cholesterol, 1.2% cholesteryl ester (calculated as oleate), 11.39% glyceride (triolein) and 4.59% of other material.

Dried liver after alcoholic extraction. Dried liver (3 kg.) was extracted exhaustively in a Soxhlet apparatus with alcohol, followed by ether, each for one working day. The final residue was dried by electric fan at room temperature and finally for a short time in an electric oven at 105°. This was used for diets IV and V.

Dried liver after aqueous extraction. Dried liver (4.5 kg.) was extracted several times with boiling water. The combined filtrate (16.5 l.) was evaporated *in vacuo* to a syrup which was used in diet VII. The liver residue was dried at 105° and was used for diet III.

Dried liver after alcoholic followed by aqueous extraction. The dried liver exhaustively extracted with alcohol as above was then extracted with boiling water as described and the residue after drying was used in diets VI and VII.

In all the diets III-VII the amount of the liver residue or extract used is that corresponding to the 75 parts of dried liver used in Exps. I and II.

In diet II the only change from diet I was the addition of two parts of choline chloride at the expense of two parts of glucose.

In diet III the material removed by aqueous extraction was replaced by glucose.

In diet IV the material removed by alcohol-ether extraction was saponified and its percentage of total fatty acids and cholesterol determined; the alcohol-ether extract was then replaced in the diet by an equivalent weight of beef dripping.

In diet V the same procedure was adopted, save that the total cholesterol present in the alcoholic extract was replaced as free cholesterol.

In diet VI the residue from the dried liver which had been extracted both with water and alcohol was fed, with the addition of the corresponding amount of beef dripping and cholesterol as in diet IV whilst in addition 2% of cholesterol was added, the aqueous extract being replaced by glucose.

In diet VII the same procedure was adopted with the addition of the aqueous extract at the expense of the additional glucose added in diet VI.

In diet VIII the animals received dried liver alone for a longer period of time.

In diet IX the animals received raw minced liver only, which was not that from which the dried liver used in all the other experiments had been prepared.

In diets X and XI the same raw liver with the addition of 0.2% and 0.4% of choline chloride respectively was used.

All the groups of rats received these diets over a period of 21 days, except groups IX, X, XI and group VIII, which were fed for 28 and 52 days respectively. After these periods of feeding the animals of groups I-VIII were guillotined, whilst those of the three groups receiving raw liver (IX, X and XI) were killed by a blow on the head. The pooled samples of the livers obtained were analysed as described by Channon and Wilkinson [1934]. The data relating to the animal side of the experiment, the food, choline, fatty acid and cholesterol intakes are recorded in Table II. The percentages of the different individual

Table II. *Weight records of animals and food intakes.*

Group No.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
No. of animals	6	6	6	6	6	6	6	4	8	8	8
Days on exp.	21	21	21	21	21	21	21	52	28	28	28
Average gain or loss (% of body wt.)	+1.3	+4.3	-1.7	+13.1	+13.8	+5.7	-2.0	+14.1	+23.8	+18.6	+22.2
Average food intake (g./rat/day):											
(a) Dry wt.	12.3	12.3	12.3	12.3	12.3	12.3	11.0	—	—	—	—
(b) Wet wt.	—	—	—	—	—	—	—	—	29.5	27.8	29.8
Average fatty acid intake (g./rat/day)	2.0	2.0	2.0	2.7	2.6	2.6	2.4	—	1.3	1.2	1.3
Average cholesterol intake (mg./rat/day)	92	92	92	—	92	338	338	110	85	80	86
Choline intake (mg./rat/day):											
Added to diet	—	214	—	—	—	—	—	—	—	56	119
Present as phosphatide	185	185	185	—	—	—	—	—	181	170	183
Total	185	399	185	—	—	—	—	—	181	226	302

Table III. *Percentage composition of the liver lipoids (g./100 g. liver).*

Group No.	Lecithin %	Free cholesterol %	Cholesteryl ester %	Glyceride %	Total %
I	4.34	0.310	1.94	3.32	9.91
II	4.56	0.302	1.82	0.90	7.58
III	4.40	0.302	2.02	1.53	8.25
IV	4.56	0.257	0.45	1.67	6.94
V	3.90	0.309	3.62	3.19	11.02
VI	4.84	0.356	5.45	3.88	14.53
VII	4.58	0.354	5.71	2.61	13.25
VIII	4.27	0.363	4.16	3.23	12.02
IX	3.26	0.294	1.85	1.84	7.24
X	3.32	0.325	1.39	1.76	6.79
XI	3.34	0.247	1.32	1.60	6.51

lipoid constituents present in the livers were calculated in the manner of Best *et al.* [1934] and the results are recorded in Table III.

DISCUSSION.

Before discussing the results, it is to be pointed out that the amount of glyceride and cholesteryl ester in the liver will depend to a considerable extent on the choline content of the diet [Best *et al.* 1934]. A further additional factor which may be involved is the protein content of the diet, for it has been found,

at least with caseinogen, that the amount of protein in the diet exercises a profound effect in controlling liver glyceride, irrespective of any effect of choline [Beeston *et al.*, 1935]. Whether this effect of the protein fraction applies to proteins other than caseinogen has yet to be determined, but it seems possible that such is the case.

(a) *The effect of dried liver.* The results for groups I and VII, in which the diet consisted mainly of dried liver, confirm the findings of Blatherwick *et al.* that a dried liver diet causes fatty livers. This fatty liver is characterised by an increase in the amount of cholesteryl esters which rise to the extent of about 1.8% after 21 days' feeding (Group I) and to 4.1% after 52 days' feeding. Such a progressive accumulation in the amount of cholesteryl esters on diets containing cholesterol has been observed before [Chanutin and Ludewig, 1933]. With this increase in cholesteryl esters there is also a rise of some 2% in the glyceride and in experiments which we have previously reported it is usual to find, even when the diet contains choline or a lipotropically active protein, the presence of rather more glyceride than normal in the liver, and the present results are similar in substance. The degree of "fat" infiltration after 3 weeks is however relatively small, for the normal figures for rat liver may be regarded as lecithin 3.8%, free cholesterol 0.3%, cholesteryl ester 0.05% and glyceride 1.0-1.5%, giving a total of 5.1-5.6% as against 9.91% of group I. This is to be connected with the high lecithin content of the liver diet and possibly with its high protein content also.

(b) *The effect of choline.* The addition of choline to the dried liver diet, so that each animal received an extra 214 mg. choline daily in group II, has had no effect on the amount of cholesteryl esters, but has lowered the glyceride content to 0.9%. This decrease in glyceride from 3.2 to 0.9% may be significant, although the significance of such a fall in the pooled livers of groups of six animals must be accepted with reserve.

(c) *The effect of alcoholic extraction of the liver.* In groups IV and V the liver used was the residue remaining after the dried liver had been extracted with alcohol. In group IV there was added beef dripping equivalent in weight to the total material removed by alcohol. This diet was thus lacking both in the phosphatide and cholesterol of diet I. The result shows quite clearly that it is the cholesterol in the liver which is mainly responsible for the fatty liver produced, for the cholesteryl ester has decreased to 0.45% as compared with 1.94% in group I. It might have been anticipated that it would have decreased to the normal value, 0.05%, and it is possible that its failure to do this depends on the difficulty of completely extracting dried liver with alcohol in bulk in the laboratory. Coincidentally with this decrease in cholesteryl ester, the glyceride falls to 1.67%, a more or less normal figure. In group V the diet was the same as in group IV, save that the amount of cholesterol in the original liver was replaced and comparison with group IV shows the result of that replacement. The cholesteryl ester fraction has increased from 0.45% to 3.62%, with a corresponding increase in the glyceride to 3.19%, showing quite clearly the effect of cholesterol in the diet in causing fatty liver production. This result seems to bring out another point, because the cholesteryl ester content, 3.62%, is considerably greater than that of the original dried liver of group I, 1.94%, and this implies that the absence of lecithin in group V is the explanation of the increase over the control figures.

(d) *The effect of the aqueous extraction of the liver.* In group III the residue after aqueous extraction was used, with the replacement of the aqueous extract as glucose. Analysis of the aqueous extract showed that the liver residue con-

tained virtually all the original cholesterol, phosphatide and glyceride. The results show that the amount of cholesteryl ester resulting is unchanged from that of the control group. This result is in contrast with that of Blatherwick *et al.*, who found that feeding water-extracted liver residue as 75 % of the diet did not result in fatty livers. It was on this evidence that they suggested the possibility that there was a water-soluble factor necessary for fatty liver production, for they pointed out that the original cholesterol of the liver was still present in the liver residue. Our results however do not provide evidence for this suggestion, because the amount of cholesteryl ester is unchanged in group III. It is true that the glyceride content of group III, 1.53 %, is some 1.8 % lower than that of the control group, but it is probable that this fall is not of significance. Six animals only were used in each group and it is our experience that it is necessary to be guarded in interpreting changes in the glyceride of this magnitude derived from the pooled tissues of such small groups.

In group VI the residue remaining after alcohol and water extraction was used as a source of liver, but the total alcoholic extract was replaced as beef dripping and its cholesterol also replaced with a further 2 % cholesterol. This diet differed therefore from the original dried liver of group I in lacking the phosphatide and water-soluble material but containing 2 % of added cholesterol. The figures in Table III show that there is increased deposition of cholesteryl ester consequent upon the inclusion of an extra 2 % of cholesterol in the diet.

Diet VII was identical with diet VI, save that the water extract was replaced, and the results are very similar to those of diet VI.

Comparisons then both of the results of group III with those of group I, and of those of group VII with those of group VI, do not seem to provide evidence for the presence of a water-soluble factor involved in cholesteryl ester deposition or for the alternative explanation mentioned in the introduction, because in all of these cases the amount of cholesteryl ester appearing in the livers is related to the cholesterol content of the diets and is uninfluenced by the presence or absence of the dietary liver aqueous extract.

One further point for notice concerning all the groups I-VIII is that the figures for lecithin in these groups are above the normal figure, which we regard as 3.7-3.8 %. This may be connected with one of two factors. It has been shown that the presence of phosphatide in diets results in the deposition of phosphatide in the liver [Rewald, 1928] and secondly that diets of high caseinogen content cause increased values for liver phosphatide [Beeston *et al.*, 1935]. Either or both these explanations may be applicable in the case of these liver diets, although the former cannot apply to groups IV and V, in which the lecithin had been removed by alcoholic extraction.

(e) *The effect of raw liver.* In groups IX, X and XI the animals received raw liver as an exclusive diet for 28 days. The results are generally similar to those of group I which received dried liver, except that the amount of glyceride is lower than in group I; this is to be correlated with the fact that whereas on both the dried and the raw liver diets, groups I and IX, the cholesterol intakes were virtually identical, 92 and 85 mg. per rat per day respectively, the fat intake was considerably lower for the raw liver group, IX, namely 1.3 g. per rat per day, as compared with 2 g. per day of the dried liver group I.

The effect of adding choline to the raw liver diets was studied in groups X and XI, in which 0.2 and 0.4 % of choline chloride were added. The increased choline intakes of groups X and XI compared with group IX were 56 and 119 mg. per rat per day respectively and this has caused no significant change in the amount of fat in the livers.

SUMMARY.

1. In confirmation of the findings of Blatherwick *et al.* it is found that the feeding of rats on diets consisting mainly of liver, either raw or dried, causes the production of fatty livers.

2. These fatty livers are characterised by the presence after 3 weeks of some 2% of cholesteryl ester with a small increase in the percentage of glyceride.

3. The addition of choline to the diet had no significant effect in decreasing the amount of fat infiltration. In this connection it is to be remembered however that the liver diets contain much lecithin, the choline of which may be exercising a maximum effect already.

4. Attempts were made to confirm the suggestion of Blatherwick *et al.* that there was a water-soluble factor present in liver, necessary for fatty liver production. The results obtained however do not support the suggestion.

The authors wish to record their thanks for the helpful advice and criticism of Prof. H. J. Channon.

Grateful acknowledgment is made to the Medical Research Council for a grant to Prof. H. J. Channon, from which the cost of this research was defrayed.

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XXI. DILATOMETRIC INVESTIGATIONS ON THE HEAT-DENATURATION OF PROTEINS. I.

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(Received November 20th, 1935.)

It has been commonly assumed that heat-denaturation of proteins is accompanied by dehydration of the protein molecules [Robertson, 1912; Pauli, 1912; 1932; Sørensen, 1925; Spiegel-Adolf, 1926; 1927; 1929; Weber and Versmold, 1931]. The last-mentioned authors showed that the "non-dissolving space" (nichtlösender Raum), i.e. such part of the system as does not dissolve an added substance of low molecular weight (sugar), decreases during denaturation. Since the "non-dissolving space" is composed of the volume of the particles and of the hydration layer, in which the water molecules are oriented under the influence of the particles, this result supports the assumption that hydration decreases during heat-denaturation.

Kruyt and de Yong [1934] tried to detect a decrease in hydration by measurements of viscosity. Their experiments however showed that the viscosity increased when protein solutions were heated to a temperature just below the coagulation point, whereas in the case of a decrease in hydration one would have expected a decrease in viscosity. It had already been shown by Loughlin and Lewis [1932] that solutions of completely denatured protein had higher viscosities than those of the natural ones. Kruyt and de Yong rightly remarked however that this increase in viscosity might be due to an increase in particle size and thus need involve no contradiction of the assumption of a simultaneous decrease of hydration. If there is a decrease in hydration during heat-denaturation it is likely that the total volume of the system will increase, since the water of the hydration layer, oriented and compressed as a result of attraction between colloidal particles and water molecules, possesses a smaller specific volume than free water. This view is supported by the observation that, if a protein swells or is dissolved in water, the total volume of the system decreases by about 6-7 ml./100 g. protein [Chick and Martin, 1913].

It is likely that the volume change during heat-denaturation will be only a small fraction of the volume decrease during the solution of the protein, just as the change of the non-dissolving space during the denaturation is only 7% of the total non-dissolving space (0.09 g. as against 1.25 g./g. protein, Weber and Versmold [1931]). Consequently for a 5% protein solution an accuracy of at least 0.002% of the total volume is necessary for qualitative detection of denaturation whilst an accuracy at least ten times as great is required for quantitative measurements.

Investigations on the volume change during heat-denaturation have been carried out by Loughlin and Lewis [1932] and by Haurowitz [1935]. They conclude that there is no volume change. Loughlin and Lewis however used comparatively dilute solutions and rather insensitive dilatometers. Haurowitz's dilatometer, although more sensitive, was of the ordinary pyknometer type. Such instruments are not reliable enough for these measurements, for some portion of their contents might easily be left adhering to the inside walls of the

top section on cooling in the thermostat after heat treatment; this would lead to false capillary readings. Moreover, Haurowitz had to introduce corrections for losses due to evaporation. Experimental errors such as these can be overcome by the employment of a mercury dilatometer as used by Heymann [1935] in studies on sol-gel transformations. The sensitivity of this dilatometer is 0.0002 % of the total volume, *i.e.* 50 times that of Haurowitz's dilatometer. Measurements on the heat-denaturation of egg albumin, serum albumin, serum globulin and zein have therefore been made with this more sensitive instrument.

EXPERIMENTAL.

The apparatus used is shown in Fig. 1. The left arm contained the protein solution and the right arm mercury. The height of the meniscus in the capillary *b* could be varied by means of tap *a*. Leakage at tap *c* was avoided by coating it with vacuum grease and by securing the cock with rubber bands. The protein solution was run in by way of *c* (2 mm. bore) using a very fine funnel. The trap *d* is designed to prevent the possible passage of any sol into the capillary, whilst bulb *e* above the capillary is a reservoir, since considerable expansion of the sol and the mercury occurs on heating.

After filling with protein sol, the dilatometer was left in an electrically regulated thermostat at 23° (constant within 0.003°) for several hours, until the mercury meniscus remained constant. Then the whole apparatus was transferred to a bath at 80° and left there for 30 min. to denature the proteins. The dilatometer was then replaced in the thermostat for about 2 hours. Prolonged temperature equilibration is necessary, because glass vessels heated to higher temperatures do not assume their original volume at once. The difference in mercury level before and after heating gives the volume change during denaturation. Usually the dilatometer was heated again to 80° for 30 min. to make sure that no further volume change occurred, *i.e.* that denaturation was complete.

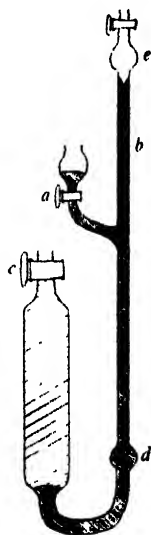


Fig. 1.

In making a determination, displacements of the mercury level of the order of 60 mm. occurred; since the capillary radius was 0.23 mm. and the total volume about 80 ml. this represents an expansion of some 0.01 % (see Table 1). Displacements of less than 1 mm. are not considered significant, so that the accuracy is about 2 % of the volume change.

Egg albumin I (Table I) was prepared from dried egg albumin (Merck) by electrodialysis with increasing potential (5 days, max. 220 v.) to precipitate the globulins. The final solution of p_H 4.9 coagulated completely on heating. As found by Pauli and Kölbl [1935], solutions at p_H 3.9 or 8.3 did not coagulate directly on heating but coagulated at once on adjustment to p_H 4.8 after heating. Egg albumin II, serum globulin and zein were very kindly supplied by Dr A. Neuberger (University College Hospital Medical School, London). The former was crystalline egg albumin prepared from egg white by accepted methods (but using Na_2SO_4 solution according to an unpublished method of Cannan) and electrodialysed for 5 days before use. The serum globulin was precipitated from ox serum by $(NH_4)_2SO_4$, and the zein was prepared essentially as described by Neuberger [1934]. The serum albumin (for which I am indebted to Dr H. Neurath) was prepared by electrodialysis of horse serum after precipitation of globulin by $(NH_4)_2SO_4$. All solutions were centrifuged from undissolved material

and used generally within a day of completing the preparation. During preparation bacterial contamination was minimised by keeping at a low temperature under a toluene-paraffin layer. p_H was adjusted with HCl, alkali or buffer solutions and measured with nitrophenol indicators. Protein concentrations were determined by dry weight of the salt-free solutions or in other cases by dry weight of heat-coagulable proteins. All dilatometric measurements showed satisfactory reproducibility.

Table I.

Protein	Conc. %	Volume of sol ml.	Volume increase		ml./100 g. protein	Behaviour on heating
			ml.	%		
Serum albumin	7.6	81	0.0123	0.0154	0.20	Coagulation
$p_H = 5.0$	5.3	80	0.0090	0.0112	0.21	
Serum globulin	9.0	81	0.0120	0.0150	0.17	Coagulation
$p_H = 5.6$						
Egg albumin I						
(a) $p_H = 4.9$	4.5	80	0.0083	0.0104	0.23	Coagulation
(b) $p_H = 3.9$	4.5	80	0.0050	0.0063	0.14	No coagulation
(c) $p_H = 8.3$	4.7	79	0.0027	0.0032	0.07	No coagulation
Egg albumin II						
$p_H = 5.8$	5.0	81	0.0105	0.0132	0.26	Coagulation
Zein in 90% alcohol	7.3	80	0	0	0	No coagulation

Table I shows the experimental results. The protein solutions which are denatured on heating all show increase in volume after denaturation. At the same time the dual nature of protein coagulation (firstly denaturation and secondly coagulation of the denatured protein) is illustrated. Only in p_H regions near the isoelectric point is denaturation accompanied by coagulation. Here the volume increase due to denaturation is between 0.20 and 0.23 ml./100 g. protein for serum albumin and egg albumin. This is about 4 g. mols. water per g. mol. egg albumin (mol. wt. 35,000) and about 8 g. mols. water per g. mol. serum albumin (mol. wt. 68,000). It is somewhat smaller for serum globulin. If egg albumin is denatured at p_H values higher or lower than the isoelectric p_H , no coagulation occurs. In this case also the volume changes are smaller, being between 35 and 70 % of the isoelectric values. These lower values cannot be due to incompleteness of denaturation at non-isoelectric p_H , since complete coagulation occurs by adjustment to isoelectric reaction after heating; this is presumptive evidence of previous complete denaturation.

Experiments have been carried out with non-purified egg albumin and "blood albumin". In these cases the volume increase was usually smaller than found with highly purified proteins. Experiments with commercial haemoglobin samples were not easily reproducible. They should be repeated with purified haemoglobin, though dilatometric investigations may present some difficulty since haemoglobin solutions contain considerable quantities of gas.

If we now consider the volume increase during heat-denaturation of isoelectric proteins (0.20–0.23 ml./100 g. protein) and compare it with the volume decrease when albumin is dissolved in water (6–7 ml./100 g. protein) we notice that the former volume change is only about 3–4 % of the latter. We may therefore assume that the decrease in hydration during denaturation is only a small percentage of the total hydration. It should however be borne in mind that there is no simple relation between the volume change and the actual change of the

number of water molecules in the attractional sphere of a protein molecule, since the volume change does not depend only on the number of water molecules influenced by the protein particle, but also on the intensity of the attractive forces.

CONCLUSIONS.

Although many chemical and physical changes during heat denaturation of proteins have been investigated, it is still difficult to give a consistent account of the nature of this important process. Therefore existing theories and opinions will only be briefly examined in this paper. Several authors [Lepeschkin, 1922, 1, 2; 1923; Wu and Wu, 1923] have assumed that heat-denaturation is accompanied by hydrolysis. It is interesting in this connection that sulphhydryl groups appear during denaturation [Hopkins, 1930]. The fact that the volume increases is however not in accordance with the existence of such hydrolytic reactions, since these processes would be connected with an increase in hydration and consequently a decrease in volume [Rona and Fischgold, 1933; Sreenivasaya *et al.*, 1934]. If such processes occurred, we should have to assume that their influence on the volume must be more than compensated by processes which bring about a volume change in the opposite direction.

Such processes have been assumed for a long time [Robertson, 1912; Pauli, 1912; 1932]. According to these authors heat-denaturation is connected with the formation of peptide bonds from —COOH and —NH_2 groups. Cohn *et al.* [1933] assume rather that —COOH groups and —NH_2 groups come into each other's sphere of attraction (*i.e.* that the zwitterionic character of the protein becomes less pronounced [Pauli, 1931]), and in this way acquire a lowered attraction for water dipoles (electrostriction). All these processes are presumably accompanied by decrease in hydration and increase in volume and their existence is thus in good agreement with the experiments of this paper. Away from the isoelectric point, where the zwitterionic character is less marked in the native protein, it is likely that a smaller volume change will occur during denaturation. This is also in agreement with the experimental results.

Several authors have discussed the possibility that denaturation results from a change of intermolecular polarisation consequent upon a change of position of some groups of the protein [Wu *et al.*, 1931; Haurowitz, 1935]. Astbury and Lomax [1935] conclude from X-ray investigations that the polypeptide chains become more regularly arranged in the molecule during denaturation. The number of such possibilities is however so great that it is difficult to foresee their effect on the volume.

SUMMARY.

The heat-denaturation of several proteins has been investigated with a sensitive dilatometer, the accuracy of which was 0.0002 % of the total volume. In all cases heat denaturation was accompanied by a volume increase which amounted to 0.20–0.23 ml./100 g. protein in the case of isoelectric proteins. If non-isoelectric proteins were used the volume increase was smaller. It has been assumed that this volume increase is caused by a decrease in hydration, which is however only a small percentage of the total hydration. Several theories of the heat-denaturation have been discussed on the basis of the experiments of this paper.

I wish to express my thanks to Prof. F. G. Donnan and to Prof. H. Freundlich for their constant encouragement and interest.

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XXII. AN INVESTIGATION OF THE ACCURACY OF THE BARCROFT DIFFERENTIAL MANOMETER IN RESPIRATION STUDIES.

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(Received November 30th, 1935.)

ALTHOUGH the Barcroft apparatus is used extensively for the study of respiration in biological fluids, we have found no published work in which the accuracy of the results obtained has been submitted to a critical examination. This is somewhat surprising when it is considered that the Barcroft apparatus is employed so frequently for comparative work where the interpretation is often founded on small differences.

In view of these considerations and from a knowledge of a certain variability in results from experiments made in triplicate as recommended by Wooldridge and Standfast [1936], it appeared desirable to submit the results obtained by use of the Barcroft technique to a statistical examination. Our work has shown that the use of four, or even of three, manometers usually suffices to give a mean with a reasonably low standard error. For qualitative work serious objection can hardly be raised against duplicate experiments, but a single experiment cannot be recommended, for it may give a dubious result which cannot be checked effectively.

The results from the following five series of experiments formed the basis for the statistical work, but use has been made also of the extensive data collected by one of us (W. R. W.) in collaboration with Mr A. F. B. Standfast. In these experiments the usual technique was followed, the manometric cups containing a total volume of liquid of 3 ml. In the following brief description only the active reagents present in the cups are mentioned.

Series I. Left-hand cup: *Pseudomonas fluorescens*; right-hand cup: *Ps. fluorescens* in *M*/40 glucose solutions. All apparatus and reagents sterilised before use.

Series II. As in Series I, but apparatus and reagents not sterilised before use.

Series III. Left-hand cup: *Pseudomonas fluorescens* + *Bacterium coli*; right-hand cup: *Ps. fluorescens* + *Bact. coli* in *M*/40 glucose solution.

Series IV. Left-hand cup: tap water; right-hand cup: crude sewage.

Series V. Left-hand cup: activated sludge; right-hand cup: sludge + crude sewage (Seitz-filtered).

Table I.

Series	Barcroft manometers used	Results rejected*	Duration of experiment in hours	Mean at end of experiment in μ l.	% standard error at end of experiment
I	14	1	9.0	175 (standard error 4.71)	2.7
II	17	2	5.0	179 (" 4.42)	2.5
III	16	2	9.5	177 (" 5.75)	3.2
IV	16	2	8.0	180 (" 6.16)	3.3
V	15	1	8.0	570 (" 10.67)	1.9

* Rejections were made on the basis of anomalous pressure difference-time curves, as explained below.

The mixtures or liquids in all cups were buffered to p_H 7.4 and temperature variations during any one experiment were slight, rarely exceeding 1° .

Readings were taken at half-hourly intervals, and experiments were continued for at least 8 hours, except those in Series II, which were terminated after 5 hours. A summary of the results is given in Table I.

A criterion for the acceptance or rejection of results.

Occasionally results from replicate experiments with the Barcroft apparatus may be so divergent that it appears advisable to reject one or more of them when calculating the mean. Sometimes, investigation at the conclusion of an experiment reveals the cause of such aberrant results (*e.g.* a leak, condensation of fluid in the manometer tube *etc.*), but in many instances no explanation is forthcoming and the rejection of a result suspected of being erroneous becomes an arbitrary procedure. We have therefore sought a criterion whereby the acceptance or rejection of a result is freed from personal bias.

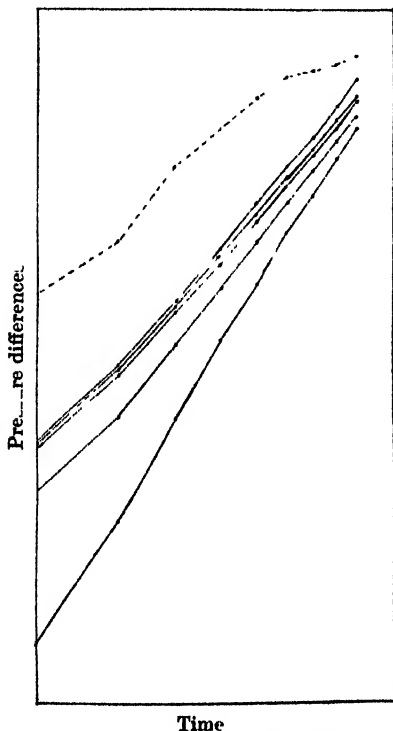


Fig. 1a. Plot of pressure differences against time on double logarithmic paper (Series II).

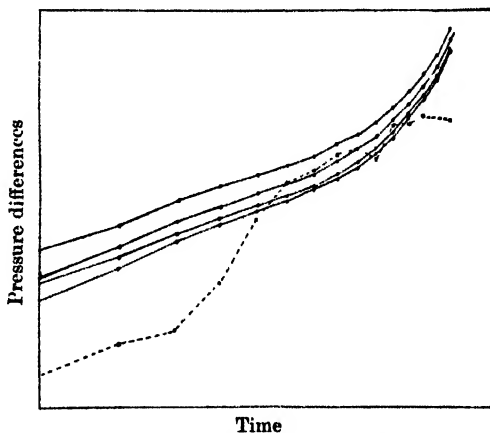


Fig. 1b. Plot of pressure differences against time on double logarithmic paper (Series IV).

In a general way it may be said that provided that the temperature remains reasonably constant, the course of oxygen absorption in the Barcroft cups tends to approximate to the equation $Y = Ft^m$, where Y is the pressure difference between the left- and right-hand cups after time t , and F and m are constants [cf. Corbet, 1934, 1]. Thus the plot of the pressure difference against the time elapsed on double logarithmic paper approaches a straight line. Under some conditions however, particularly when gas uptake is rapid, the values of F and

m change so rapidly that the resulting curves tend to be parabolic rather than linear: nevertheless, the curves from replicate experiments are more or less parallel although, in general, they tend to converge with increasing time. We found that the pressure difference-time curves of experiments known to be faulty did not follow their fellows, but usually differed so radically in character that such experiments could be detected immediately by this means and legitimately rejected. Series of curves from two sets of identical experiments are depicted in Figs. 1*a* and 1*b*: it will be seen that both series contain a faulty experiment (dotted curve) which can be omitted justifiably from further consideration. Occasionally, the plot on double logarithmic paper reveals parallel but widely spaced curves. This implies a high standard error and one may feel tempted to reject the divergent results: we do not consider that such a procedure can be upheld, however, for then the question which results have to be rejected becomes a matter of personal opinion. The occasional appearance of these presumably normal results demonstrates the importance of not drawing conclusions from a single experiment.

The question of the acceptance or rejection of results suspected of being faulty is one of considerable importance. In fact we might go so far as to say that the degree of precision possible in experiments with these differential manometers depends on the detection and elimination of such faulty experiments. On the other hand, rejection of readings suspected of being too high or low for insufficient reasons will produce results whose accuracy is fictitious rather than real.

Calculation of the mean.

The usual statistical methods of assessing the accuracy of experimental data are based on the assumption that these observations are normally distributed about their mean and, if such is the case with results derived from experiments with the Barcroft apparatus, it is correct to express the mean of the results by dividing their sum by the total number of experiments. On the other hand, if the logarithms of the values, and not the values themselves, are normally distributed (as is the case with parallel plate counts of soil and milk samples [Corbet, 1934, 2]), then it is preferable to determine the geometric mean.

To decide conclusively whether the values from a series of replicate Barcroft experiments are normally distributed requires more results than are usually available but we have examined the question in the light of the results obtained from the five series of experiments mentioned above. The normality of the distributions was tested by calculation of Pearson's constants β_1 and β_2 (see Table II). For a symmetrical distribution β_1 is zero whilst β_2 approximates to 3, β_2 being greater or less according to whether the curve is leptokurtic or platykurtic. In Table II are included results found by Cook and Stephenson [1928] for oxygen absorption by bacteria in $M/200$ glucose solution; this experiment differs from those of Series I-V in that the oxidation was carried to completion.

Table II.

Series	Number	Actual values		Logarithms of actual values	
		β_1	β_2	β_1	β_2
I	13	0.62	1.96	0.21	1.94
II	15	0.16	1.85	0.092	2.42
III	14	0.21	2.02	0.17	2.12
IV	14	0.24	1.76	0.83	1.98
V	14	0.01	2.18	1.33	2.24
Cook and Stephenson [1928]	28	0.23	2.45	0.23	2.45

Deviations of β_1 and β_2 from zero and 3 respectively can only be regarded as significant if the respective values of $\sqrt{\beta_1}$ and of β_2 are $> 2 \times 0.65$ and 2×1.31 for 14 results, and 2×0.46 and 2×0.93 for 28 results [Tippett, 1931]. It will be seen from the table that, although the distributions of the results of Series I–III are not significantly asymmetrical, the logarithms of the actual values suggest a more normal distribution; on the other hand, the actual values from Series IV and V, rather than their logarithms, are normally distributed. In no case, however, is the divergence from zero and 3 of the β constants sufficient to justify working with logarithmic rather than arithmetic values.

The standard error in Barcroft experiments.

The standard deviation is a measure of the distribution of the individual results about the mean value: it is usual to express this deviation in terms of the standard error and to regard as statistically significant any results which differ from the mean by more than twice the standard error. The standard error and standard deviation are related in the manner shown by the following expression:

$$\text{standard error} = \frac{\sigma}{\sqrt{N}} = \sqrt{\frac{\sum (x - \bar{x})^2}{N(N-1)}},$$

where σ is the standard deviation, $\sum (x - \bar{x})^2$ is the sum of the squares of the individual deviations from the mean and N the number of results.

It is desirable to ascertain how the standard error varies with duration of the experiment, the degree of replication and the magnitude of the mean. The standard error remains constant during the course of a single experiment only if the curves representing the oxygen absorption with time are parallel to one another: when these curves converge, diverge or cross, the standard error changes accordingly. Although occasionally instances of divergence or intersection are encountered, results from a large number of experiments show that these curves tend to converge as time proceeds, and so, as a general rule, the standard error diminishes as the experiment progresses.

The plot of the percentage standard error against time in the five series of experiments referred to (Table III and Fig. 2) shows that the standard error approaches a constant value which, for all practical purposes, is attained within 5 or 6 hours. The result of summing the curves 1, III, IV and V (II was continued for 5 hours only) and taking the mean percentage standard errors is depicted also in Fig. 2. The curves for Series I–III (pure cultures of bacteria) show initially a high standard error which falls rapidly: in Series IV and V (sewage and sludge) the standard errors are almost constant throughout. The explanation of the difference in character between these two types of curves may be that, whilst the introduction of an active strain of bacteria into a sterile medium with an abundance of a readily oxidisable compound was associated with initial growth phases of varying duration in different manometers, with material containing a micro-population already in a condition approaching equilibrium, no very abrupt changes occurred and the gas absorption in the manometers was much more uniform. On the other hand, in a series of experiments in which the oxygen absorption by soil was measured in presence of KOH, the curve representing the decrease in percentage standard error with time was almost identical with that for Series III.

It is evident from Fig. 2 that, as a general rule, a consistently low percentage standard error can be expected only after an experiment has been in progress for not less than 5 hours.

Table III. *Variation of standard error with duration of experiment.*

Series	% standard error after			
	1 hour	3 hours	4.5 hours	6.5 hours
I	8.58	4.10	3.12	2.76
II	7.32	3.54	2.68	—
III	26.06	9.40	5.35	3.71
IV	3.70	3.18	3.24	3.22
V	2.48	1.78	1.47	1.75

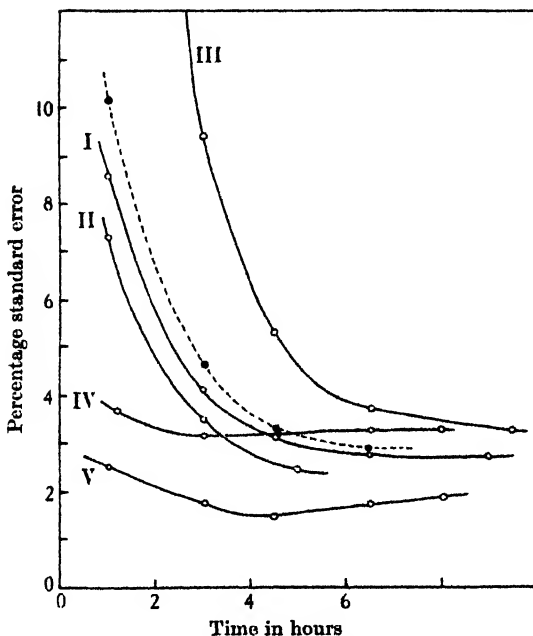


Fig. 2. Variation in standard error with duration of experiment. The dotted curve represents the mean values for the curves of Series I, III, IV and V.

The calculated variation in the percentage standard error with the degree of replication found in our five series of experiments is shown in Table IV and Fig. 3.

The figures and graphs show that a standard error of about 5% would be obtained with 4 manometers for $6\frac{1}{2}$ hours and with 5 manometers for $4\frac{1}{2}$ hours; duplicate results gave a standard error under 10% for periods over 4 hours. It will be recalled that faulty results were eliminated, so as a general practice it is advisable to prepare one more replicate than the number considered to give a sufficiently low standard error.

There is some evidence suggesting that the percentage standard error may increase slightly as the magnitude of the mean decreases; if this is so, it appears to be too slight to have any practical significance. It is possible, too, that the percentage standard error may decrease somewhat as the experimental temperature is raised. Most of our data refer to work carried out at 20° , so we are unable to test this point adequately.

Table IV. *Variation of standard error with degree of replication.*

(a) After $4\frac{1}{2}$ hours

(a) After 4½ hours		No. of replicate experiments						
	15	14	13	8	6	4	3	2
	% standard error found			% standard error calculated				
Series								
I	—	—	3.12	3.83	4.42	5.41	6.25	7.65
II	2.68	—	—	3.55	4.09	5.02	5.79	7.09
III	—	5.35	—	6.82	7.87	9.62	11.13	13.64
IV	—	3.24	—	4.13	4.76	5.83	6.74	8.25
V	—	1.47	—	1.88	2.17	2.66	3.07	3.75
Mean	3.17			4.04	4.66	5.71	6.60	8.08

(b) After $6\frac{1}{2}$ hours

Series	No. of replicate experiments						
	14	13	8	6	4	3	2
	% standard error found		% standard error calculated				
I	—	2.76	3.38	3.90	4.78	5.52	6.76
III	3.71	—	4.72	5.45	6.67	7.70	9.44
IV	3.22	—	4.11	4.74	5.81	6.70	8.21
V	1.75	—	2.23	2.57	3.15	3.64	4.46
Mean	2.86		3.61	4.17	5.10	5.89	7.22

(c)

Cook and Stephenson [1928] (to completion)	No. of replicate experiments					
	28	14	7	4	3	2
	% standard error found	% standard error calculated				
	1.73	2.39	3.24	4.48	5.18	6.34

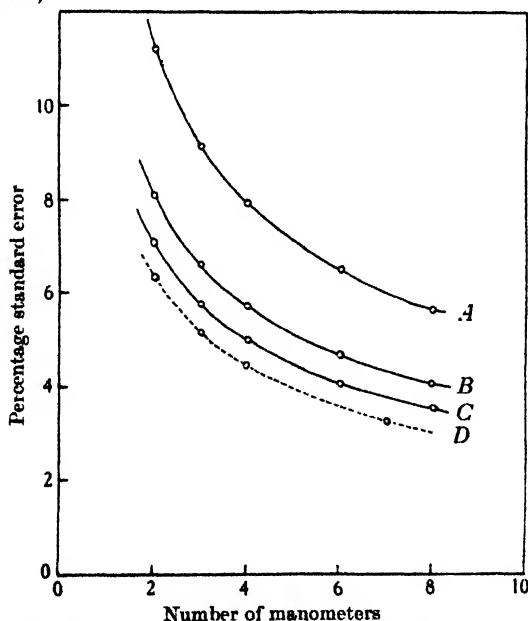


Fig. 3. Variation in standard error with replication. The curves A, B and C represent the mean percentage standard errors for Series I-V after $3\frac{1}{2}$, $4\frac{1}{2}$ and $6\frac{1}{2}$ hours respectively. Results from the data of Cook and Stephenson (experiments carried to completion) are expressed by the curve D.

Examination of the significance of differences between means.

Often in experiments with the Barcroft apparatus it is necessary to compare results of two or more series of experiments: with large differences the interpretation to be placed upon them usually presents no difficulty, but the precise significance to be attached to small differences can be decided only after statistical examination of the data.

When the standard errors of two means are known, the probability of the difference between them arising by chance can be estimated and, if the odds against are sufficiently great, this difference is accepted as real. Usually, it is satisfactory to work on the $P=0.05$ level of significance; that is, to regard as significant all differences which would occur by chance less often than once in 20 times.

Dixon and Elliott [1930] concluded that the absorption of carbon dioxide in the Barcroft manometer is carried out more efficiently in presence of a roll of filter-paper soaked in KOH than by KOH solution alone. We have tested this conclusion in a replicated experiment and find that, under the particular conditions of our experiments, there is no significant difference between the two methods.

Two parallel sets of experiments, in which oxidation of the sterilised crude sewage was effected by the addition of mixed sewage bacteria, and in which carbon dioxide absorption was effected either by KOH solution or by KOH-saturated papers placed in the central tube of the Barcroft cups, gave the following results:

(a) KOH solution: mean of 6 experiments = 84.3 (s.e. 1.18) μ l.

(b) KOH-saturated papers: mean of 8 experiments = 80.0 (s.e. 3.36) μ l.

Fisher's t [1934] is calculated from the formula

$$t = \frac{(\bar{x} - \bar{x}')}{s \sqrt{\frac{1}{N} + \frac{1}{N'}}},$$

where \bar{x} and \bar{x}' are the respective means derived from N and N' observations and s is obtained from the relation

$$s^2 = \frac{\sum (x - \bar{x})^2 + \sum (x' - \bar{x}')^2}{(N - 1) + (N' - 1)},$$

the terms $\sum (x - \bar{x})^2$ and $\sum (x' - \bar{x}')^2$ representing the respective sums of the squares of the individual deviations from the means.

For the results of the experiments (a) and (b) we have

$$s^2 = \frac{(42 + 632)}{(5 + 7)} = 56.17,$$

and

$$t = \frac{(84.3 - 80.0)}{\sqrt{56.17} \cdot \sqrt{\frac{1}{6} + \frac{1}{8}}} = 1.063.$$

Entering the table for t at $n = [(N - 1) + (N' - 1)]$ (that is, at $n = 12$), it is found that P is greater than 0.3 for $t = 1.063$, implying that the difference between the means of (a) and (b) can arise by chance about one in 3 times and so is without significance.

A second example involving differences between three sets of experiments is of some importance from the viewpoint of sewage purification. A crude sewage was sterilised by autoclaving and was subsequently aerated in equal quantities with washed activated sludge and with the same sludge previously autoclaved.

After 3 hours' aeration the mixtures were centrifuged and the supernatants examined in Barcroft manometers to see whether their rates of oxidation in presence of activated sludge were different from that of the original autoclaved sewage; in other words, to test whether 3 hours' aeration with either of the sludges effected any reduction in the content of oxidisable matter of the original sewage. The following results were obtained:

(a) Autoclaved sewage: mean of 4 experiments 234 (s.e. 25.2) μ l.

(b) Autoclaved sewage after aeration with activated sludge: mean of 4 experiments 81 (s.e. 17.4) μ l.

(c) Autoclaved sewage after aeration with autoclaved sludge: mean of 4 experiments 179 (s.e. 4.79) μ l.

From the results of experiments (a) and (b) we have $t=5.00$ and $n=6$, and P is found to be considerably less than 0.01 and so the difference between the means of experiments (a) and (b) is highly significant. That is to say, aeration of the sewage with activated sludge has significantly reduced its content of oxidisable matter.

For the means of (a) and (c), however, $t=2.147$ corresponding to a value of P between 0.1 and 0.05 and showing that the difference between these means can arise by chance once in 10 to 20 times. Thus, although aeration of a sewage with an autoclaved sludge appears to reduce its oxidisable matter somewhat, the results obtained hardly permit definite conclusions to be drawn. But, in this example, the standard error of (a) is unusually high, thus precluding any precise deductions from being made regarding a comparatively small difference between (a) and (c). The standard error might have been reduced by higher replication or continuance of the experiment for a longer period of time.

In the same way, with the means of (b) and (c), t is 5.425 for which, at $n=6$, P is much less than 0.01 and so the difference is highly significant. Thus, even if the autoclaved sludge had produced some purification of the sewage such purification is very considerably increased by aeration with a normal activated sludge.

These two examples should suffice to illustrate the value of the use of the Barcroft technique in the way which we suggest.

SUMMARY.

A statistical study of results obtained by the use of the Barcroft apparatus for respiration experiments has shown that deductions from single, or even duplicate, experiments should be accepted with caution and that a higher degree of replication is desirable.

On the basis of our conclusions we have adopted the following procedure.

(a) Experiments are planned in triplicate, quadruplicate, or even with higher replication, according to the standard error expected (see Fig. 3) and the probable magnitude of the differences to be examined.

(b) Experiments are continued for a period appropriate to the number of replicates used so as to give a standard error not appreciably greater than 5% (see Fig. 2).

(c) The actual values of the pressure differences found at hourly intervals are plotted on double logarithmic paper and results of any experiments giving anomalous curves (see text) are rejected without further examination.

(d) After conversion of the pressure differences into volumes (by multiplication of the manometer factors), the arithmetic means are found, the standard errors calculated, and the difference between the means investigated as shown on p. 138.

If such a procedure be adopted the Barcroft differential manometer can be employed usefully in the investigation of small or large differences in many biological oxidations.

This investigation was carried out as part of the programme of the Water Pollution Board of the Department of Scientific and Industrial Research. Our thanks are due to our colleague, Dr J. O. Irwin, for advice on certain statistical matters.

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XXIII. THE USE OF THE BARCROFT DIFFERENTIAL MANOMETER IN THE ESTIMATION OF THE OXYGEN ABSORPTION OF SEWAGE.

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OUR earlier investigations on the purification of sewage by the activated sludge process [Wooldridge and Standfast, 1932; 1933; Wooldridge, 1933] led us to two main conclusions. Firstly, we concluded that probably the most important factor in sewage purification was a series of catalysed oxidation-reduction reactions determined by bacterial enzymes, present in either living or dead bacterial cells, or liberated by them into the fluid of the reacting system. Secondly we formed the opinion that neither of the two tests investigated was entirely satisfactory for use in a systematic study of the conditions influencing the purification of sewage by the activated sludge process; *e.g.* the technique for the determination of B.O.D. necessitates the incubation of many portions of the same sample for various periods of time. Considerable improvement could clearly be effected if the progress of oxygen absorption could be followed continuously on one sample. With this object in view experiments have been carried out on the measurement of the rate of oxidation of sewage in a Barcroft micro-respirometer.

Are gases other than carbon dioxide produced when sewage or activated sludge is shaken with excess of air?

Before it was possible to apply the manometric method to a study of sewage oxidations it was necessary to ascertain whether the quantity of gases other than oxygen and carbon dioxide which might be present changed during such experiments. It is well known that under anaerobic conditions such gases as hydrogen, hydrogen sulphide and methane are readily produced from sewage or sludge mixtures. It was thought probable however that, when these oxidations were effected in the presence of excess of air or oxygen, especially when the fluid was kept saturated with oxygen by continuous agitation, none of these gases would be produced. These conditions of oxygen saturation are secured in the Barcroft apparatus, wherein 3 ml. of the sewage are shaken in cups of a total capacity of 35-40 ml. In order to examine whether these gases are found in this type of experiment a larger proportion, *viz.* 150 ml. of sewage, sludge or sewage-sludge mixture was shaken with air in 500 ml. bottles for 6 hours, the gas above the mixtures being analysed after 20 min. shaking open to the air and after periods of shaking of 3 and 6 hours in sealed bottles subsequent to this. The gas samples were examined in a Haldane gas apparatus modified for the detection of combustible gases [Haldane, 1920], but in no case could any measurable quantity of combustible gas be detected. The results given in Table 1 represent one of a duplicate series in which all duplicates agreed.

Table I. *Examination of formation of combustible gas.*

The samples were shaken in 500 ml. bottles stoppered with rubber bungs containing inlet and outlet tubes to facilitate sampling the gas. Three bottles were employed in each series of experiments. The sewage or mixtures shaken consisted of (a) 150 ml. crude sewage, (b) 50 ml. thick suspension of sludge *plus* 100 ml. distilled water and (c) 50 ml. of sludge *plus* 100 ml. crude sewage. After 20 min. preliminary shaking with the tubes open to the air 75 ml. samples of gas were taken with all the usual precautions to avoid sampling errors. Similar samples were taken at the end of 3- and 6-hour shaking periods.

	Hours of shaking	Volume of gas analysed	Volume of gas after CO ₂ abs.	Volume of gas after ignition	Volume of gas after second CO ₂ abs.	Volume of combustible gas
Crude sewage	0	9.445	9.420	9.420	9.420	0
	3	9.420	9.375	9.375	9.375	0
	6	9.420	9.370	9.370	9.370	0
Sludge	0	9.290	9.283	9.283	9.283	0
	3	9.320	9.300	9.300	9.300	0
	6	9.408	9.389	9.389	9.389	0
Sludge + crude sewage	0	9.350	9.330	9.330	9.330	0
	3	9.470	9.288	9.288	9.288	0
	6	9.450	9.111	9.111	9.111	0

The production of gaseous hydrogen sulphide in these manometric sewage experiments was tested for by placing 0.3 ml. of lead acetate solution in small side arms of several cups containing sewage liquor, sludge or sewage-sludge mixtures. The respirometers were then closed and shaken at 24° for 21 hours. Even at the end of this time there was no sign of blackening of the acetate solution, indicating that the production of hydrogen sulphide under these aerobic conditions had been negligible.

It is known that under certain conditions free nitrogen is liberated from soil and hence its possible evolution during the process of aerobic oxidation of sewages cannot be neglected in an investigation dependent upon changes in the volume of the gas in the apparatus. In order to test this possibility 30 ml. quantities of sewage, washed sludge or a mixture of sewage and sludge were shaken for 5 hours in sealed bottles of 400 ml. capacity at 20° with excess air. The concentrations of sewage, sludge and air in the bottles were thus practically identical with those generally employed in the Barcroft cups. The total nitrogen contents of the mixtures were estimated by the Kjeldahl process both before and after the aerobic shaking. The experiments were made in triplicate and, as will be seen from Table II, no change in the nitrogen content of the fluid was observed

Table II. *Examination of the production of nitrogen.*

Experiment	Composition of solution in bottles				% of nitrogen in solution	
	Sludge ml.	Crude sewage ml.	Distilled water ml.	Buffer ml.	Initial g.	Final g.
1	10	0	15	5	0.028	0.028
2	0	15	10	5	0.005	0.005
3	10	15	0	5	0.033	0.033

and therefore it appears that gaseous nitrogen was not liberated. It may be concluded that under the ordinary conditions of a few hours' shaking with excess air in a Barcroft manometer sewage systems do not normally liberate free nitrogen.

Nevertheless we are not justified in concluding from this experiment that free nitrogen can never be liberated from oxidising sewage systems, *e.g.* one containing a very high content of nitrate.

The oxygen absorption by sewage measured manometrically.

If equal volumes of a sewage and water are put respectively into the right- and left-hand cups of a Barcroft differential manometer and the apparatus is shaken in a water-bath at constant temperature then the difference that develops in the levels of the menisci of the manometer fluid will enable us continuously to determine the oxygen absorption of the sewage at the temperature of the experiment. In order to absorb CO_2 0.3 ml. of 10 % KOH solution is placed in the inner tube of all cups. This has been a routine procedure unless otherwise stated. In the majority of our experiments it has been found that 1 ml. of a crude sewage or 3 ml. of a sewage effluent are generally suitable, but the total volume of liquid in each cup is always made up to 3 ml. with buffer or water. The results given in the tables or figures indicate the oxygen absorption per ml. of sewage or mixture. The temperature of the experiments has not always been the same but it has generally been 24° , the lowest temperature at which the baths could be kept constant throughout the year. All manometers are shaken in the bath for 15–20 min. with the manometers open to the air to enable the system to reach temperature equilibrium before closing the taps. The factor for each manometer has to be worked out for each temperature, but once the factor is known for any particular temperature the actual change in volume of the gas in the right-hand cup can be readily obtained by multiplying the difference in levels of the manometer fluid by this factor. The results are most readily expressed in $\mu\text{l.}$ of oxygen absorbed by 1 ml. of sewage. If it is required to conform to current practice in sewage analysis and express results in terms of parts of oxygen absorbed by 100,000 parts of sewage then the result expressed in $\mu\text{l.}$ of oxygen absorbed per ml. of sewage must be multiplied by the factor 0.143.

For strong sewages 1 ml. is sufficient to give very definite results, but for weak sewages 2 or 3 ml. should be used. The results given in Table III illustrate the oxygen absorptions per ml. of several different sewages over a period of 5 days;

Table III. *Oxygen absorption of various sewages.*

No.	Hours										Parts per 10 ⁵ calc. 120 hrs.
	3	6	9	12	21	27	45	69	78	102	120
1	45.6	71	89	98	133	155	220	313	339	400	443
2	26.4	71	156	—	262	295	405	484	498	534	560
3	43.9	—	—	—	204	235	296	398	—	—	586
4	18.4	67	—	—	160	190	248	298	—	355	387
5	23.6	52	100	—	194	220	270	306	323	368	401
6	10	27	36	—	82	92	121	149	165	—	177
7	—	62	—	98	120	133	165	192	202	220	240

the oxygen absorption was measured in $\mu\text{l.}$ but the last column contains the calculated 5-day figures representing parts of oxygen absorbed per 100,000 parts of sewage. Thus the sewages examined absorbed oxygen in 5 days equivalent to 25 to 85 parts per 100,000. Although there is some variation in rates of oxidation of the different samples they all show the general characteristic of a more rapid oxidation in the earlier (10–20) than in the later hours. In none of the experiments, however, had the absorption of oxygen completely ceased at the end of the 5 days.

It is evident from these results that the differential manometer can be used to follow the oxygen uptake of crude sewage but it remains to be seen whether differences such as those existing between sewages or effluents taken at different stages in the plant of a sewage disposal works can be readily demonstrated. The results given in Table IV show that this is possible and that the oxygen absorbed

Table IV. *Oxygen absorption of sewage at different stages of purification.*

The figures show the oxygen absorption in μ l. at N.T.P. per ml. of sewage preparation. The right-hand cups contained the sewage preparations unbuffered and diluted to 3 ml., and the left cups 3 ml. water. KOH paper was present to absorb CO_2 . The amounts used were: crude sewage (unfiltered) 1 ml.; effluent from sedimentation tank 2 ml.; effluent from filter 3 ml.; final effluent after land treatment 3 ml.

Hours shaken in Barcroft app. ...	1	3	5	10	25	45	70	91	120
Crude sewage (unfiltered)	10.9	23.6	43.5	112	212	270	308	346	400
Sedimentation tank effluent	4.9	12.5	22.9	48.8	78	101	118	135	153
Filter bed effluent	2.4	4.0	4.5	4.9	8.1	11.7	15.4	17.8	23.4
Final effluent after land treatment	0.6	1.0	1.3	2.0	3.7	6.0	8.8	10.7	16.3

in any given time by the four samples is always in the expected order, viz. crude sewage, sedimentation tank effluent, effluent from filters, final effluent. Even within a few hours an approximate idea of the relative strengths of the sewages and sewage effluents is clearly demonstrable. It should be noted that the final effluent shows a definitely measurable, although small, uptake of oxygen when 3 ml. of the effluent are used.

In view of this small absorption of oxygen the absorption values with a number of different effluents were measured. Table V represents the results obtained

Table V. *The oxygen absorption of various sewage effluents in the Barcroft manometer.*

Except for those in the last column the figures show the oxygen absorption in μ l. at N.T.P. per ml. of effluent. The right-hand cups contained 3 ml. of effluent and the left-hand cups water. CO_2 was absorbed by potash.

Effluent	Temp. °C.	Hours									Parts per 10 ⁵ calc. 120 hrs.
		3	5	20	30	48	54	72	96	120	
I	22	—	2.3	3.0	4.5	6.0	8.4	9.0	—	22.0	3.1
II	22	2.2	3.0	6.5	8.3	10.4	11.4	13.6	21.2	24.3	3.5
III	21	2.4	—	5.3	5.9	7.7	8.9	10.8	15.4	22.0	3.1
IV	21	0.7	—	4.3	5.8	6.8	7.2	8.6	10.9	13.1	1.9
V	21	1.6	—	4.5	5.0	5.3	5.4	7.3	11.7	14.8	2.1
VI	21	1.9	—	5.9	7.2	9.3	9.5	13.2	19.7	27.4	3.9
VII	21	1.2	—	3.9	4.7	6.3	6.4	6.9	7.9	8.2	1.2
IX	24	4.0	4.5	6.0	10.8	11.8	—	15.6	18.9	23.0	3.3
XIII	24	—	—	6.6	—	13.2	14.8	19.9	29.1	36.0	5.1
VIII	21	0.6	1.0	3.3	4.0	6.6	7.1	8.5	12.8	16.3	2.3

with ten effluents (not all "satisfactory" final effluents) and it will be seen that after 5 days the absorption of oxygen per ml. of effluent varied between 8.2 and 36.0 μ l.

By this method it is easy to distinguish the oxygen absorbing capacity of a strong sewage from that of an effluent after examination over a period of 5 days, but it is worth considering whether it is possible to form a conclusion from measurements at an earlier stage. Tables III and V show that with both sewages and effluents the rate of absorption of oxygen under these conditions is greatest in the first few hours. In spite of this, however, the values for the two groups are well separated almost from the beginning. In 20 hours all the sewages have absorbed more than ten times the amount absorbed by even the strongest treated effluent, whilst in 6 hours the same ratio generally holds although at this point the weakest sewage had absorbed six times as much oxygen as the strongest effluent. It is possible then that the careful following of the rate of oxygen absorption over a period as short as 6 hours might give something more than an approximate idea of the condition of the sewage, especially when a number of manometers are used.

Comparison of manometric and dissolved oxygen (B.O.D.) methods.

Before using the manometric method to any great extent either in research or in routine analytical work it is advisable to compare the oxygen absorption values obtained by the manometric and by the dissolved oxygen (B.O.D.) methods. For this purpose a number of sewages and effluents have been examined simultaneously by the two methods, the incubation being at the same temperature, *viz.* 22°. In the tests for B.O.D. care was taken to ensure that a satisfactory dilution was used, the sewage usually being incubated at two different dilutions. Estimations by both methods were made in duplicate, the illustrative examples being taken from experiments where duplicates were in close agreement. The results are shown in Table VI.

Table VI. *Comparison of manometric and B.O.D. values for various sewages.*

Manometric method (M.) as in previous table but expressed in parts per 10⁶. B.O.D. values obtained by usual method. Temperature 22°.

Sample no.	1		2		3		4		5 days	
	M.	B.O.D.	M.	B.O.D.	M.	B.O.D.	M.	B.O.D.	M.	B.O.D.
1	31.2	28.2	44.6	35.5	59.8	44.6	—	—	84	65
2	16.2	14.5	25.8	19.0	32.9	22.3	—	—	41	29
3	12.7	3.9	16.5	9.9	20.9	13.4	28.4	18.9	35	21
4	17.3	6.2	20.9	10.7	24.8	12.0	27.0	15.6	29	18
5	7.6	2.6	10.6	4.9	12.6	5.9	14.0	7.0	16	8
6	2.4	1.4	3.7	2.3	4.6	2.7	5.3	3.1	5.7	3.4
7	1.5	0.5	2.1	1.4	3.0	1.8	3.7	2.1	4.6	3.4
8	1.0	0.9	1.5	1.6	1.9	1.7	—	—	3.5	3
9	1.0	0.4	1.3	1.0	1.9	1.4	2.8	1.9	4.8	2.6
10	1.2	0.8	2.4	1.4	2.8	1.5	3.1	1.9	3.6	2.1
11	0.5	0.7	0.9	0.8	1.3	0.8	—	—	3.1	2.0
12	0.6	0.3	0.9	0.6	1.7	1.5	2.0	1.7	2.6	1.9
13	0.7	0	0.8	0.7	1.1	0.7	1.6	0.8	2.5	1.0
14	0.5	0.2	1.0	0.5	1.3	0.5	1.7	0.6	2.8	0.8
15	0.7	0.1	1.0	0.2	1.3	0.3	1.5	0.7	2.2	0.8

It will be noticed that in every instance the oxygen absorption values obtained by the manometric method are greater than the corresponding results obtained by the B.O.D. method; in many instances the B.O.D. value is about a third less than the manometric figure but comparison of the values for many sewages shows that no definite ratio between the two values appears to exist. A result such as this is to be expected since the conditions of the two methods are by no

means identical. In the manometric method the fluid is kept saturated with oxygen and so a more rapid oxidation might be expected than with a method wherein the tension of dissolved oxygen falls continuously as the period of examination extends. Furthermore the actual concentrations of the sewage are usually quite different in the two methods and carbon dioxide is continuously absorbed in the one method and not in the other. With such differences it is possible that dissimilar flora would develop in a period of 5 days.

Table VII. *Oxygen absorption by a sewage at different dilutions.*

Results expressed in $\mu\text{l.}$ at N.T.P. per ml. C.S. Conditions as in previous tables.

Sewage dilution	Hours									
	1	2	5½	16	24	44	64	92	112	120
100	15	27.5	60	98	124	163	187	213	236	240
50	7.1	13.9	22	45.4	59	77	90	104	120	122
20	1.5	4.1	8.3	14.5	22.3	32.1	36	42.4	46	47
10	0.2	1.0	2.7	6.5	9.0	15	18.3	22	22	23

DISCUSSION.

Whilst it has been shown that oxygen absorption by sewage and sewage effluents can be followed manometrically, there remains to be considered the advisability of employing this method when so much previous work has been done with the test for biochemical oxygen demand in 5 days as the standard of reference. It is perhaps unfortunate that the manometric method furnishes results somewhat higher than those obtained by the older method and it is certain that before the new method can be generally applied, *e.g.* in testing whether an effluent is satisfactory, it will be necessary to make an exhaustive comparison of the values obtained by the two methods. Such a comparison might show that contrary to the deduction we have made from the few results given in this paper, a sufficiently satisfactory conversion of one set of results into the other can in fact be secured by employing some simple factor. In any case it is probable that a much shorter period of examination than five days may allow a satisfactory judgment of a sewage or an effluent to be formed using the manometric method. Although we have not carried out a sufficiently extensive examination, we would nevertheless suggest tentatively that a good effluent might be classified as one which does not give an oxygen absorption value as great as one part per 10^5 parts of effluent after shaking for a period of 24 hours in a Barcroft manometer at 22° . This conclusion is suggested by the results given in Table VI, where it will be noticed that effluents that show values by the ordinary B.O.D. test of less than 2 parts per 10^5 fall into this category. We are inclined to think, however, that were this method further explored a more definite standard of reference for judging effluents would be arrived at and one which might require a considerably shorter period, say 6 hours. The manometric technique however suffers from certain drawbacks when the examination is extended over very long periods. The most troublesome of these is that after several days drops of moisture may be deposited in the manometric tubes, especially if the latter are at a temperature considerably below that of the cups. In any case experiments should always be made at least in duplicate and when quantitative comparisons are desired a larger number of determinations is necessary. If after further experiment the method should prove of use in analytical work then the manometric scales could be calibrated and marked to read directly in parts of oxygen absorbed per 10^5

parts of the sewage provided that the temperature and the amount of sewage placed in the cups are standardised for all determinations. For the examination of effluents it might prove an advantage to use quantities larger than 3 ml. with or without a modification of the size of the cups. In any event the technique of the method and the determination of the constants for the apparatus, as described by Dixon [1934], are generally applicable. One advantage which the manometric method has over the ordinary B.O.D. test is that the sewages and effluents can be examined without the great dilution which is often otherwise necessary when examining a strong sewage by the B.O.D. test. Further with the B.O.D. test the sewage is often made up in two dilutions and then it is frequently found, even when each bottle has excess of dissolved oxygen at the end of 5 days that the final B.O.D. values are considerably different. With the manometric methods this dilution error, which may be dependent on the oxygen tension, is largely avoided since the fluid is fully saturated with oxygen throughout the experiment and the sewage is never diluted more than 1 in 3. Even if samples of the same sewage are used at widely different dilutions experiment has shown that the oxygen absorptions measured manometrically are near the values expected (see Table VII).

For the purposes of research into the mechanism of sewage purification processes however the manometric method has several important advantages over the B.O.D. test. An obvious advantage is that much smaller quantities of sewage can be examined. Further by this method the process of oxygen absorption can be followed continuously and the effect of an alteration in experimental conditions, such as change in temperature, the addition of some reagent *etc.*, can be readily ascertained. In this way considerable saving in time is effected. Thus substances may be added to the sewage that could not be used before since they interfered with the reagents employed in the subsequent determination of the dissolved oxygen. The fact that each manometer possesses two cups enables a condition to be varied on one side and not on the other, when its effect, if any, will be shown by the manometric reading. In this way, for example, we may investigate amongst other things, the effect of the addition of activated sludge to sewage. In fact when we have, say, 1 ml. of a sludge suspension and 2 ml. of sewage in the cup of a manometer, we have under fairly well controlled conditions, a system that is akin to the "activated sludge process" in miniature, for the presence of excess air and the shaking of the apparatus together form ideal conditions for such purification to take place. For this reason, among the others we have discussed, we have adopted this technique as a serviceable method for investigating sewage purification.

SUMMARY.

1. It is shown that the oxygen absorption from air by sewage and sewage effluents can be followed manometrically using the Barcroft differential manometer.
2. The oxygen absorption for any particular sewage is usually greater when measured manometrically than when it is estimated by the usual test for biochemical oxygen demand.
3. Under the aerobic conditions of the Barcroft apparatus such gases as hydrogen, methane, hydrogen sulphide and nitrogen do not appear to be liberated during oxidation of ordinary sewage systems.
4. The reasons for adopting the manometric method are discussed, and tentative suggestions are made for its use in routine sewage analysis.

This investigation was carried out as part of the programme of research of the Water Pollution Board of the Department of Scientific and Industrial Research. Our thanks are due to the Borough Engineer of Finchley for the regular supply of the samples of sewage used in this work.

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XXIV. SOME EXPERIMENTS ON THE OXIDATION OF SLUDGE AND SLUDGE- SEWAGE SYSTEMS.

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It has been shown [Wooldridge, 1933], by means of experiments with methylene blue in Thunberg vacuum tubes, that sewage sludge, especially activated sludge, is capable of effecting a number of biological oxidations, including that of sterile crude sewage. In the present paper this activity of activated sludge (hereinafter simply called "sludge") is more fully examined using the Barcroft differential manometer as described in the previous paper [Wooldridge and Standfast, 1936]. In that paper it was shown that this apparatus could be used with advantage to follow the oxidation of non-sterile sewage. In the present paper the technique is extended to the examination of sludge and mixtures of sludge and sewage.

The oxidation of sludge.

Typical examples of the autoxidation of sludge as measured in a Barcroft apparatus are given in Table I. In Table II are given the results of a comparison of the rates of oxygen absorption by a sludge and by a sewage, the two experiments being run concurrently. It will be seen that in this particular experiment both the sludge (0.5 ml. of the suspension) and the sewage (2.5 ml.) appeared to be completely oxidised in approximately the same time, *viz.* 110 hours. The sludge, however, required a very much greater quantity of oxygen than that

Table I. *Oxygen absorption by sludge as measured by the
Barcroft manometer.*

Each cup of the apparatus contained 1 ml. of phosphate buffer, p_H 7.4, and 0.3 ml. of 10% KOH solution in the small inner chamber. The right-hand cup contained 0.5 ml. of a suspension of washed sludge and 1.5 ml. of water. The left-hand cup contained 2 ml. of water. The sludge was prepared by collecting a known quantity from an experimental tank, washing it thrice by centrifuging and re-suspending in water and finally making it up to a strength of three times that used in the tank. Finally, to facilitate accurate measurement the suspension was passed through muslin to remove exceptionally large particles. After the cups had been filled the apparatus was assembled, placed in a bath at 24° and shaken for 20 min. with free access to the outside air. The stopcocks were then closed and readings of the height of the manometric fluid taken at once and subsequently at the end of every hour. The figures give the oxygen absorption in μ l. per ml. of sludge suspension.

Hours											
Sludge	1	2	3	4	5	6	10	22	30	43	48
1	34	54	71	86	101	118	160	249	310	412	457
2	97	135	170	199	227	252	333	572	784	972	—
3	37	70	99	122	147	171	237	377	459	547	572
4	71	138	180	223	253	—	375	542	—	—	—
5	58	96	137	173	—	227	295	469	—	—	—

Table II. *The oxygen absorption by crude sewage and by sludge in the presence of phosphate buffer, p_H 8.*

The sewage or sludge was placed in right-hand cups only. The figures give the oxygen absorption in μ l. at N.T.P. per ml. of the sewage or sludge. Other conditions as in Table I.

	Hours at 24°											
	1	2	4	6	10	20	35	50	70	90	110	144
Crude sewage	10	16	23	29	35	45	60	78	94	105	115	115
Sludge	88	140	230	312	420	620	940	1240	1506	1580	1600	1600

required by the sewage. The experiments show that the Barcroft manometer may be used to follow the oxygen absorption by sludge, and that the course of the experiment may be followed to completion if suitable quantities of material are chosen. As might be expected this uptake of oxygen is directly proportional to the amount of sludge present (Table III). Hence, at least within the limits of the concentrations chosen, the effect of dilution on the oxidation of the sludge is negligible. Experimental details are given in the table headings.

Table III. *Oxygen absorption by different quantities of washed sludge.*

Experimental details were the same as for Table I except that no buffer was added to any of the cups.

Volume of sludge suspension ml.	Oxygen absorption in μ l. after							
	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	210 min.	240 min.
0.5	6.0	10.7	14.1	18.8	24.1	28	31.7	36.5
1.0	11.5	20.3	28.7	36	45.5	53.2	61.1	71.6
1.5	17.3	30.1	45.8	53	66.2	79.2	90	103.8
2.0	22.8	40.8	58.1	72.8	91.9	108	122	142
2.5	28.3	50.2	72	92.2	115	136	155	180
3.0	34.4	62.1	88.7	113	141	166	188	218

The oxidation of crude sewage in the presence of sludge.

If the oxidation of a mixture of sludge with crude sewage is followed in a Barcroft manometer concurrently with similar experiments in which the same quantities of sludge or of crude sewage are allowed to oxidise separately, then it is found that the oxygen absorption recorded by the mixture is greater than the sum of the absorptions of the two constituents taken separately. Hence in the presence of each other the rate of oxidation would appear to be increased (Table IV). In Table IV the figures represent the differences in the quantities of oxygen absorbed in the two cups of each respirometer after the experiments had been in progress for different periods of time. After the respirometer had been shaken at 22° for 6 hours, the quantity of oxygen absorbed in the right-hand cup exceeded that in the left by an amount equivalent to 60 μ l. at 0° and 760 mm. In this particular experiment the difference represents the quantity of oxygen absorbed by the sewage as the left-hand cup contained only water. Experiment B was similar to experiment A but 1 ml. of sludge and 2 ml. of water were placed in the right-hand cup. The quantity of oxygen absorbed by this amount of sludge in 6 hours was 227 μ l. If a mixture of sewage and sludge absorbed the same quantity of oxygen as the sum of the amounts absorbed by the same volumes of sewage and sludge examined separately, the result for experiment C after 6 hours should have been 287 μ l. Actually the uptake of oxygen in 6 hours by a mixture of these quantities of sewage and sludge was much greater, viz. 605 μ l. This shows

Table IV. *Absorption of oxygen from air at 22° by crude sewage, activated sludge and mixture of sewage and sludge.*

Nett absorption of O_2 in $\mu\text{l.}$ at N.T.P. The contents of the cups of each manometer are indicated below; the abbreviations CS., Sl. and W. signify respectively 2 ml. crude sewage, 1 ml. sludge and water *ad* 3 ml. CO_2 was absorbed in all cups by the usual method of adding 10% KOH to the inner cup.

Exp.	Contents of Barcroft cups		Period in hours						
	Left	Right	1	2	3	4	5	6	
A	W.	CS. + W.	16	32	46	49	55	60	
B	W.	Sl. + W.	51	97	137	175	204	227	
C	W.	Sl. + CS.	116	229	336	446	532	605	
D	Sl. + W.	Sl. + CS.	81	148	210	279	325	391	
E	CS. + W.	CS. + Sl.	102	211	305	408	484	555	
	(A + B)		67	129	183	224	259	287	
	C - (A + B)		49	100	153	222	273	318	
	E - B		51	114	168	233	280	328	
	D - A		65	116	164	230	270	331	

that by mixing crude sewage and activated sludge the rates of oxidation by air of the sewage or sludge or of both are greatly accelerated. Without this acceleration the figures for experiment E should be the same as those for B, and the results for D (where the sludge oxidation is balanced) should equal those for A. In the lower part of Table IV the sum of the results for experiments A and B is given and then the increased oxygen absorption due to mixing the sewage and sludge is calculated from the experimental results in three ways, *viz.* $C - (A + B)$, $E - B$ and $D - A$. The good agreement between these figures lends considerable support to the deductions made. A comparison of these figures with the totals for A and B shows that during the first 6 hours and with the particular samples employed the rate of oxidation of a mixture of sewage and sludge by air was about twice the sum of the rates for the constituents agitated with air separately.

That this increased oxygen absorption is mainly an acceleration of the normal oxygen uptake is brought out by the experimental results given in Table V. Here are compared the rates of oxygen absorption by a crude sewage alone and

Table V. *The acceleration of sewage oxidation in the presence of sludge.*

In these experiments 0.5 ml. of phosphate buffer, p_H 7.0, was present in each cup and 2 ml. and 0.5 ml. of sewage or sludge suspension respectively in the cups indicated. The total volume of the liquid contents of each cup was made up to 3 ml. with water. CO_2 was absorbed in each cup by 10% KOH. The temperature of the bath was 24°. In the Table below W., B., CS. and Sl. signify water, buffer, crude sewage and sludge respectively. The figures indicate the oxygen absorption in $\mu\text{l.}$ at N.T.P.

Contents of left cup	Contents of right cup	Hours										
		1	2	3	4	6	8	9	21	28	33	40
B. + W.	CS. + B. + W.	5	12	17	22	40	70	85	125	135	140	155
Sl. + B. + W.	Sl. + CS. + B.	41	62	80	94	112	125	130	160	162	162	162
B. + W.	Sl. + B. + W.	50	69	87	100	124	143	155	271	361	420	455
CS. + B. + W.	CS. + Sl. + B.	70	115	140	160	181	186	188	304	375	430	465

by a crude sewage-sludge mixture with an equal amount of the sludge alone in the other cup. Thus after 4 hours' agitation at 22° the experiment with sewage alone shows an oxygen utilisation of 22 $\mu\text{l.}$ whilst in the other experiment the difference between the oxygen absorption of the sewage in the presence of sludge and that due to the sludge alone is 94 $\mu\text{l.}$ After 40 hours however, the figures are in

comparatively close agreement, *viz.* 155 μ l. and 162 μ l. respectively. A similar result can be demonstrated by a second pair of experiments in which are compared the oxygen absorption values for sludge alone and a for system sludge *plus* crude sewage with crude sewage alone in the opposite cup. In these experiments the 4-hour figures were 100 μ l. and 160 μ l. and the 40-hour figures 455 and 465 μ l. Thus it is shown that although the final total amount of oxygen absorbed by a sewage and a sludge oxidising separately will together equal the amount absorbed by the constituents mixed together yet the rate of oxidation is greatly accelerated by admixture. The rate of the acceleration is, as expected, dependent to some extent on the amount of sludge present in the mixture. The rate of oxidation however, is not necessarily directly proportional to the concentration of sludge as will be seen from the results given in Table VI, although the increased oxidation

Table VI. *Oxidation of sterile crude sewage in the presence of different quantities of sludge.*

In these experiments, which were carried out in triplicate, the sludge suspension was placed in both cups and the volume of the liquid made up to 2.5 ml. with water. 0.5 ml. of crude sewage, sterilised by passage through a Seitz filter, was placed in the side arm of the right-hand cup and 0.5 ml. of water in the side arm of the left-hand cup. After the preliminary 20 min. shaking the contents of the side arms were tipped into the cups, and oxidation of the sewage began. CO_2 was absorbed by KOH in all cups. The figures below represent the absorption of oxygen in μ l. at S.T.P. in the times at the head of the columns.

Amount of sludge ml.	Hours						
	1	2	3	4½	8½	9½	18½
0.5	8	10.5	14.5	19.5	40	43.5	99.5
1.0	11.7	17.3	22.3	33	68	75	111
1.5	17.7	28	39	57	102	105	119
2.0	21.3	35	52	73	116	119	128
2.5	27.3	43	63	91	127	128	141

for each additional 0.5 ml. quantity of sludge suspension is approximately constant in the early stages of the experiment. Clearly many other factors, such as concentration of substrate *etc.*, have some effect upon the rate of oxidation.

The oxidation of sewage effluents in the presence of sludge.

When sewage effluent is allowed to oxidise in a Barcroft apparatus, either alone or in the presence of sludge, results are obtained similar to those given in Table IV with crude sewage. Thus in parallel series of experiments the oxygen absorption by a mixture of an effluent and sludge exceeds the sum of the values obtained by allowing the effluent and the sludge to oxidise separately. The actual values are less with effluent than with crude sewage but the increased oxidation is nevertheless definite. Furthermore, the increase may be calculated in three different ways from the five experiments and, as will be seen from the last three lines of Table VII, the three results are approximately the same.

If the oxidation of an effluent in the presence of sludge is allowed to continue for a few days it will not necessarily reach a final maximum value but generally continues to increase slowly so that the oxygen absorption value of the effluent alone remains very much below the value reached in the presence of the sludge even when this oxidation is continued for long periods. This result differs from that obtained with a crude sewage, the values for which in the absence and in the presence of sludge generally tended to approach each other after two or more days of oxidation. In the presence of sludge, however, the oxygen absorption value

Table VII. *Absorption of oxygen from air at 22° by sewage effluent, activated sludge and mixtures of effluent and sludge.*

Experimental details as in Table IV. The abbreviations Eff., Sl. and W. signify 2 ml. effluent, 1 ml. sludge and water *ad* 3 ml. respectively.

Exp.	Contents of Barcroft cups		Period in hours					
	Left	Right	1	2	3	4	5	6
A	W.	Eff. + W.	11	17	17	19	19	19
B	W.	Sl. + W.	26	47	65	80	94	103
C	W.	Sl. + Eff.	55	86	107	125	140	151
D	Sl. + W.	Sl. + Eff.	31	44	49	50	52	53
E	Eff. + W.	Eff. + Sl.	47	76	94	112	127	137
		A + B	37	64	82	99	113	122
		C - (A + B)	18	22	25	26	27	29
		E - B	21	29	29	32	33	34
		D - A	20	27	32	31	33	34

for effluents still remains considerably lower than the value obtained within the same time by most crude sewages. Whether this oxygen uptake is entirely due to the more rapid oxidation of the constituents of the effluent by the sludge or is due to changes in the bacterial flora or to some other cause cannot at present be stated. It is noticeable with some effluents that the rate of oxidation may become practically stationary for a period of hours and subsequently increase at a greater rate again. This will be seen from an examination of the results given in Table VIII where the rates of oxygen absorption of four effluents collected from four sewage works are given over a period of 94 hours both in the presence and in the absence of sludge.

Table VIII. *The oxygen absorption values of various sewage effluents as ascertained in the presence or in the absence of sludge.*

The figures at the head of the columns represent hours and the figures in the body of the table the oxygen absorption in μ l. per ml. of effluent at N.T.P.; 2 ml. of effluent were present in all right-hand cups. Where sludge was added 1 ml. was present in both cups of the manometer. The total volume of fluid in each cup was made up to 3 ml. with water. CO_2 was absorbed by 10% KOH in the usual way. The effluents used were the final effluents obtained from four different sewage disposal works in the London area.

		Hours								
Effluent	Sludge	3	19	25	43	51	67	75	91	94
C.	-	2.4	5.3	5.4	7.1	8.2	9.0	11.3	14.5	15
C	+	14.5	48.7	59	62	63.5	71	79	95	98
Ed.	-	0.7	4.1	4.9	6.3	7.1	8.1	8.9	10	10.7
Ed.	+	8.8	44	54	66	70.5	82	91	106	109
En.	-	1.6	4.8	4.8	5.1	5.3	6.9	9.8	10.8	11.5
En.	+	19	62	73	76	76	80	85	102	105
F.B.	-	1.9	5.9	7	7.5	9.4	12	14.2	19.6	19.7
F.B.	+	23	69.5	86	104	108	118	122	138	140

Nevertheless there would appear to be certain advantages to be gained from allowing the oxidation of a sewage or effluent to proceed in the presence of sludge, advantages which are of value in research and possibly in tests at a works. By this means a measure of the difference between a crude sewage and a sewage effluent can be shown in a few hours, *e.g.* a comparison of the oxygen absorption values in Table IV for crude sewage alone and in Table VII for effluent alone

shows that within the first 6 hours the difference between the two although evident was not very great. When however the sewage and effluent were allowed to oxidise in the presence of sludge their rates of oxidation were greatly increased and within 6 hours the increased oxygen absorption values (the autoxidation of the sludge being allowed for by its presence in the other cup of the Barcroft apparatus) showed a marked difference.

In order that the method may distinguish between the oxidisabilities of two or more effluents, however, it is preferable that there should be sufficient replicates, and that differences should only be regarded as real if the results are statistically significant. With slow rates of oxygen absorption some variation in individual results may be obtained; *e.g.* two effluents from the experimental activated sludge tank were collected, one after the sewage had been aerated for $4\frac{1}{2}$ hours, and the other after 7 hours' aeration; these effluents, in 2 ml. quantities, were put up in the right-hand cups of Barcroft manometers in the presence of washed activated sludge, the left-hand cups containing a similar quantity of sludge without the effluent. After 5 hours the oxygen absorption values of the six manometers containing the $4\frac{1}{2}$ -hour effluent were 61, 92, 68, 83, 50 and $66\mu\text{l.}$, giving a mean of $70 \pm 6.20\mu\text{l.}$ The corresponding values for the 7-hour effluent were 30, 20, 41, 51, 49, $41\mu\text{l.}$, giving a mean of $39 \pm 4.81\mu\text{l.}$ From these results [*v. Corbet and Wooldridge, 1936*] the value for Fisher's *t* is found to be 3.955, and hence *P* is less than 0.01. The difference between the two series of results is therefore highly significant, showing that a definite further purification of the sewage took place even after the preliminary period of $4\frac{1}{2}$ hours' aeration with sludge. For this examination less than 20 ml. of the effluent were required, whilst for a 5-day B.O.D. test a much larger quantity is required even to do a single test. The results of such a single test will probably be unreliable for weak effluents; thus the following results, which suggest that the 7-hour effluent was less pure than the $4\frac{1}{2}$ -hour effluent, were obtained. For the $4\frac{1}{2}$ -hour effluent the 5-day B.O.D. value = 1.15 parts per 10^5 and for the 7-hour effluent = 1.55. These results might vary considerably with higher replication but they suggest that the B.O.D. test is probably insufficiently sensitive to measure accurately differences between the oxidisability of good effluents.

If the method were developed as a routine procedure it would be an advantage to use a standard sludge, say one that alone would bring about an absorption of oxygen of $100\mu\text{l.}$ per hour per unit quantity. Work along these lines might make it possible to utilise this as a standard method for the rapid examination of sewage and effluents and so enable a table to be drawn up which would prove of value in assessing the quality of the sewage or effluent. There would be no difficulty in carrying out a short preliminary test with any particular sludge suspension to ascertain the quantity required to give the standard rate of autoxidation; but variations in the nature and so in the enzymic activity of the sludge from day to day might prove a difficulty in practice unless a sludge powder could be prepared in a dry stable form. Such ideas however, before general application in the field, require further careful investigation. For purposes of research into the mechanism of the activated sludge process of sewage purification however it will be seen that the method described in this paper allows continuous observation, under controlled conditions, of the process on a very small scale, with measurements of the rates of oxygen absorption.

SUMMARY.

1. It is shown that the rate of absorption of oxygen by an activated sewage sludge may be followed in a Barcroft differential manometer.

2. The initial rate of oxidation of a crude sewage or of a sewage effluent is greatly increased by the presence of sludge although the ultimate oxygen absorption value is scarcely influenced by this factor.

3. Using this method of following the oxidation of a sewage or effluent in the presence of sludge it is shown that marked differences in the oxygen demands of various sewages are demonstrated within a few hours.

This investigation was carried out as part of the programme of research of the Water Pollution Board of the Department of Scientific and Industrial Research. Our thanks are due to the Borough Engineer of Finchley for the regular supply of the samples of sewage used in this work.

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XXV. CERTAIN FACTORS THAT INFLUENCE THE RATE OF ACTIVATED SLUDGE AND SEWAGE OXIDATIONS.

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WE have shown in previous papers [Wooldridge and Standfast, 1936, 1, 2] that oxygen absorption by sewage, by sludge or by sewage-sludge mixtures may be conveniently studied by means of Barcroft differential manometers and that small but significant differences may be investigated by replicated experiments [Corbet and Wooldridge, 1936]. The present paper describes the results of experiments on the effects of various conditions on the rates of oxidation.

Effect of temperature on the oxidation of sewage.

As sewage oxidation normally takes place somewhat below atmospheric temperature it is possible, if the oxidation is due to the presence of highly specialised flora or fauna, that its rate might be greater or less at higher temperatures such as 30° or 37° than at, say, 18°. Samples of many different sewages and effluents have been studied at 18°, 22°, and 30° or 37°, and all, with the exception of a few effluents, have shown increased rate of absorption of oxygen with rise in temperature. No direct comparison, however, was made on any one sample of its rates of oxidation at 30° and 37°. The results in Table I illustrate the relative

Table I. *Effect of temperature on the oxidation of sewage.*

2 ml. of crude sewage and 1 ml. of phosphate buffer, p_H 7.4, were placed in the right-hand cup of each Barcroft manometer, with buffer and water in the left-hand cups. Carbon dioxide was absorbed by placing 10% potash in the inner tubes sealed into the bottom of the cups. The results given are the mean in every case of six experiments simultaneously made, the manometers being shaken in a bath of water kept at the stated temperature. The figures heading the columns represent the number of hours after the experiment began and those in the body of the table give the corresponding oxygen uptake (expressed in μ l. at N.T.P.) by 1 ml. of sewage.

Sewage	Temp. C.	Hours									
		3	7	24	27	31	48	51	67½	104	120
I	18	15	52	159	170	184	228	232	258	296	328
	22	19	73	179	190	205	257	265	297	363	388
	37	56	128	231	248	265	312	320	350	405	423
II	18	10	31	86	92	99	126	130	148	170	177
	22	12	38	89	95	104	133	137	159	191	202
	37	24	61	116	125	133	166	169	190	230	245
III	18	2	3.3	5.5	6	7	8.6	8.7	11	18	21
	22	2	3.8	7.1	7.8	8.1	10.4	10.9	12.9	20.3	24.3
	37	3	4.6	8.3	8.6	9.2	11.4	11.5	16.4	27.3	33

rates at 18°, 22° and 37° of three sewage liquors of different strength; sewage III was a treated effluent. With all three samples similar results were obtained. We have on occasion, however, found that with a satisfactory sewage effluent the

rate of oxidation at 37° or 30° may be slightly lower than that at 22°. The explanation of this has not been found but it may be associated with the nitrate content of the effluent. Although it has been shown that sewage oxidation will generally proceed more rapidly at 30° or 37° we have carried out most of our work at 22° or 24° as we wished to study the problem at temperatures nearer to those generally maintained in sewage purification plants.

Effect of hydrogen ion concentration on sewage oxidation.

Samples of sewage were adjusted to various p_H values between 4.0 and 10.0 by the addition of small amounts of acid or alkali and their rates of oxidation followed in a Barcroft apparatus. Results so obtained seemed to suggest that the oxidation took place readily between p_H 6.0 and 9.0, with perhaps an optimum between 7.0 and 8.0, but unfortunately the p_H values did not remain constant and no definite conclusion from these experiments is warranted. Table II gives

Table II. *Effect of p_H on oxygen absorption by crude sewage.*

The figures in the table represent the oxygen absorption in μ l. at N.T.P. by 1 ml. of crude sewage over a period of 9 hours when shaken with excess air in a Barcroft apparatus at varying p_H values, the latter being kept constant by the addition of buffer of the required p_H . The CO_2 evolved was absorbed by KOH papers. The final p_H values were examined and found to be identical with the original. Each right-hand cup contained 2 ml. of sewage and 1 ml. of the buffer and each left-hand cup 2 ml. of water and 1 ml. of buffer. Temperature 22°.

Buffer	p_H	Hours						
		1	2	3	4	5	6	9
Phthalate	4.5	0	0	0	0	0	0	1.6
	5.2	0	0	0	0	0.2	1.3	2.0
	6.0	2.7	5.5	8.1	14.0	20.2	41.2	61.0
Phosphate	6.1	4.3	8.2	12.0	22.6	35.0	53.7	102.0
	6.6	6.0	11.9	20.0	32.3	49.0	71.0	131.0
	7.3	10.1	20.0	34.0	54.0	77.0	93.3	133.0
	8.0	9.0	19.3	31.3	46.5	66.4	85.3	126.0
Borate	8.0	4.5	7.9	12.1	15.9	19.8	25.9	55.0
	8.4	1.6	9.7	10.3	14.7	19.3	25.0	57.3
	9.0	4.5	8.4	11.9	15.4	18.8	24.1	52.3
	9.6	4.4	7.5	9.7	11.2	12.9	14.2	23.0

results obtained with samples of a crude sewage buffered at various p_H levels. It will be seen that practically no oxidation occurs below p_H 5.0 and that it begins to fall off again above 9.0. Between p_H 6.6 and 9.0 there is fairly rapid oxidation with perhaps a maximum rate at about 7.3. The presence of phthalate or of borate buffer appears to inhibit somewhat the oxidation (*cf.* the values obtained at p_H 6.0 and at 8.0 in the presence of phosphate and phthalate or borate buffers).

When a similar experiment is carried out with a suspension of washed activated sludge the oxidation of the sludge is found to be fairly vigorous over a wider range, *viz.* between p_H 6.0 and 10.0, whilst its inhibition at p_H 5.0 is only slight. Little oxidation usually occurs at p_H 4.0 or at values above 12.0. Illustrative results are given in Table III. It will be noticed that the phthalate and borate inhibitions are less marked with the oxidation of sludge than with that of sewage. Allowing for the borate inhibition there would appear to be no significant optimum p_H for the oxidation of sludge between 6.0 and 9.0.

The rate of oxidation of a sewage at various concentrations of hydrogen ions in the presence of sludge, however, resembles that for sewage alone, for the

Table III. *Effect of p_H on the oxidation of sludge.*

The figures in the table represent the oxygen absorption in μ l. at N.T.P. of 1 ml. of sludge over a period of 5 hours when shaken with excess air in a Barcroft apparatus at different p_H values, the latter being kept constant by the addition of buffers. The CO_2 evolved was absorbed by KOH. The sludge was prepared by washing several times with water a sample obtained from the activated sludge tank and filtering the final suspension through muslin. The last cup had 1 ml. $N/10$ NaOH added instead of buffer. The p_H was determined again at the end of the experiment and found to be unchanged (no test was made on the $N/30$ NaOH). Each right-hand cup contained 1 ml. of sludge suspension, 1 ml. of the required buffer and 1 ml. water, the left-hand cups containing only the buffer and water. Temperature 22° .

Buffer	p_H	Hours				
		1	2	3	4	5
Phthalate	5.0	22.5	46.2	65.4	83.0	101.0
"	6.0	44.7	74.7	97.5	118.5	134.5
Phosphate	6.0	47.3	78.2	100.3	121.3	138.2
"	7.0	32.0	60.3	83.6	103.9	122.4
"	8.0	36.0	68.3	92.8	115.0	135.9
Borate	8.0	29.5	58.0	80.0	100.5	118.0
"	9.0	31.7	63.4	85.0	106.0	124.0
"	10.0	30.4	60.0	84.0	95.0	108.0
$N/30$ NaOH between	12.0-13.0	5.1	7.2	11.7	14.4	16.2

Table IV. *Effect of p_H on the oxidation of crude sewage in the presence of washed sludge.*

The figures in this table represent the difference between the oxygen absorbed by a sludge-sewage mixture and that absorbed by the sludge alone at different p_H values. Results are given in μ l. of O_2 at N.T.P. The experiments were made at 24° . Each right-hand cup contained 0.5 ml. of a washed sludge suspension previously adjusted to the required p_H , 0.5 ml. of the requisite buffer and 2 ml. of a Seitz-filtered crude sewage, also adjusted to the necessary p_H . In the left-hand cup the sewage was replaced by water. The final p_H values were determined and found not to differ from the initial values. CO_2 was absorbed by KOH.

Buffer	p_H	Hours				
		1	2	3	4	5
Phthalate	4.0	0	0	0	0	0
	5.0	0.8	2.4	2.4	2.4	2.4
	6.0	4.5	17.0	33.0	55.0	81.0
Phosphate	6.0	7.9	30.0	51.5	73.0	90.8
	7.0	30.0	56.0	73.5	90.0	102.0
	8.0	26.0	49.6	64.0	83.4	92.7
Borate	8.0	15.0	24.9	35.4	49.5	66.3
	9.0	15.0	25.3	32.8	49.8	65.3
	10.0	1.2	2.7	3.9	5.7	5.7

optimum again returns to the region of p_H 7.0 (*v.* Table IV) and little or no oxidation takes place at p_H 5.0 or 10.0. The rate of oxidation is approximately constant between p_H 6.0 and 9.0 if allowance is made for the effect of borate buffer. It is perhaps worthy of notice that although the sludge itself may be undergoing oxidation to some extent at p_H 5.0 and 10.0 (see Table III) this does not appear to enable it to oxidise the sewage at these p_H values, a result which indicates that some of the substrates of washed sludge are different from those of sewage. We may conclude therefore that for the oxidation of sewage by the activated sludge process it is preferable to work at p_H values between 7.0 and 8.0, but little loss in efficiency will take place if the p_H should drop to 6.0 or rise to 9.0. Outside these values considerable loss in efficiency may be expected.

Evolution of carbon dioxide in sewage oxidations.

It is to be expected that with the oxidation of the carbonaceous constituents of sewage some carbon dioxide would be produced and in order to avoid error due to the production of this gas it is usual in work with a Barcroft differential manometer to remove the carbon dioxide from the atmosphere in the cups by placing 0.3 ml. of 10% KOH (as liquid or impregnated filter-paper) in the small inner tubes fused to the bottom of the cups. All the experiments so far reported have been carried out with KOH present, but usually without paper as there appears to be no significant difference under our experimental conditions between the two techniques for carbon dioxide absorption [Corbet and Wooldrige, 1936]. By carrying out simultaneously two sets of similar experiments, identical except that the potash is present in one set and absent from the other, a difference will be noted in the volumes of the right-hand cups of the two series, the difference being due to the presence of carbon dioxide in those cups containing no KOH. In this way, and employing the appropriate manometer factor, the actual amount of carbon dioxide produced can be calculated from the readings of the two sets of experiments. Different constants are necessary for the same manometer in the presence or absence of KOH because of the different solubilities of the gaseous contents of the cup when it contains either oxygen and nitrogen alone or oxygen, nitrogen and carbon dioxide [v. Dixon, 1934]. Tables V and VI give typical results obtained when crude sewage or a sewage-sludge

Table V. *Oxygen absorption and carbon dioxide evolution by a crude sewage at p_H 7.0.*

The results given are the mean of six different experiments run concurrently. Each left-hand cup contained 1 ml. of phosphate buffer p_H 7.0, and 2 ml. of water; each right-hand cup contained 2 ml. of crude sewage in place of the water. The O_2 absorption figures were obtained from manometers containing 10% KOH in the cups and the CO_2 figures by deducting the change in volume of right-hand cup contents when no KOH was present from the change in volume when the CO_2 was absorbed by KOH, the appropriate factors being used for estimating these volume changes. The latter are expressed in μ l. at N.T.P.

The latter are expressed in μ l. at N.T.P.

	Hours									
	1	2	3	4	5	6	9	20	21	
O_2 absorbed	20.2	33.2	44.9	59.8	78.2	100.1	147.0	218.0	220.0	
CO_2 evolved	4.5	8.9	15.0	23.7	38.0	52.3	79.7	122.0	123.0	
Ratio CO_2/O_2	0.22	0.27	0.34	0.40	0.49	0.52	0.54	0.56	0.56	

mixture is allowed to oxidise in this way. The results given in Table V show the quantity of oxygen absorbed and the amount of carbon dioxide evolved over a period of 21 hours by a crude sewage. From an examination of these results it will be seen that the ratio of the carbon dioxide produced to the oxygen utilised is much less than unity although as the oxidation proceeds the ratio appears to increase. A similar result is obtained when a sewage-sludge mixture is allowed to oxidise at varying p_H , e.g. at p_H 6.4, 7.0 or 8.0 (Table VI). These results may be due to the retention of carbon dioxide by the colloidal mixtures but a preferable explanation is that in the early stages the oxidation of the carbonaceous sewage constituents is less complete than at a later stage or that a greater proportion of the oxygen is utilised in oxidising nitrogenous or other non-carbon materials in the earlier part of the oxidation. The fact that the ratio would appear to be somewhat higher in an acid medium might be explained on the basis of a greater inhibition of non-carbonaceous oxidation, e.g. ammonia, at this p_H .

Table VI. *Oxygen absorption and carbon dioxide evolution by a sewage-sludge mixture at different p_H values.*

Each left-hand cup contained 1.5 ml. water and 1.5 ml. of the requisite phosphate buffer. In the right-hand cups the water was replaced by 1.5 ml. of a sewage-sludge mixture. The oxygen absorption values were obtained directly from the manometric readings of experiments in which the CO_2 evolved was absorbed by 10% KOH. The CO_2 evolved was obtained by deducting from the above readings those obtained from manometers in which no KOH was present. The appropriate factors for converting the readings into $\mu\text{l. gas at } 0^\circ \text{ and } 760 \text{ mm. pressure}$ were used.

p_H		Hours				
		1	2	3	4	5
6.4	O_2 absorbed	78	108	133	159	179
	CO_2 evolved	37	68	100	124	145
	Ratio CO_2/O_2	0.47	0.63	0.75	0.78	0.81
7.0	O_2 absorbed	95	143	178	212	238
	CO_2 evolved	35	72	102	133	151
	Ratio CO_2/O_2	0.37	0.50	0.57	0.64	0.63
8.0	O_2 absorbed	80	131	179	227	268
	CO_2 evolved	39	74	103	139	178
	Ratio CO_2/O_2	0.49	0.55	0.58	0.61	0.66

Saturation of activated sludge with sewage.

From experience with activated sludge plants at sewage disposal works it is known that the sludge, if it is "overworked", may become ineffective as a sewage purifying agent. This is mainly due to a supersaturation of the sludge with sewage constituents so that the addition of a further quantity of sewage cannot lead to any further increased activity on the part of the sludge, with the result that the final addition of sewage is not oxidised. In support of this view the following experiments (Table VII) made with a sludge saturated with crude sewage may be examined. Sludge was obtained from the experimental tank, washed with water, subsequently shaken with a large volume of crude sewage (about

Table VII. *Effect of the saturation of a sludge with sewage upon its ability to oxidise added sterile sewage.*

The activated sludge was saturated by shaking twelve times with a large excess of crude sewage. The sewage was sterilised by passing through a Seitz filter. In the table below Sl. = 1 ml. saturated sludge, (CS.) = 0.5 ml. sterile sewage placed initially in side arm of cup, B. = 1 ml. buffer of p_H 7.0, W. = water to make a total volume of 3 ml. and (w.) = 0.5 ml. water in side arm.

The sludges used in Exps. A and B were different samples. Carbon dioxide was absorbed by KOH in all cups. The figures heading the columns state the time, in hours, after the beginning of the experiment at which the contents of the side arm were tipped into the cups. The figures in the table represent the oxygen absorption due to the added CS. by the saturated sludge in the first 2 hours after tipping. The volume of oxygen absorbed is expressed in $\mu\text{l. at } 0^\circ \text{ and } 760 \text{ mm.}$, the figures of Exp. A having been corrected for the oxygen uptake due to the sludge alone in the same period. In Exp. B the control unsaturated sludge figure gives the rate at which the initial sludge, before treatment with excess of sewage, oxidised sterile sewage.

Exp.	Contents of cups		Hours of shaking before tipping					
	Left	Right	0	1	2	3	4	5
A	B. + W. + (CS.)	Sl. + B. + W. + (CS.)	71	74	82	89	114	—
B	Sl. + B. + W. + (w.)	Sl. + B. + W. + (CS.)	37	44	51	56.5	62	71
	Control unsaturated sludge		75	—	—	—	—	—

ten times its own volume) and allowed to sediment. The supernatant sewage was siphoned off and fresh sewage added. This process was repeated twelve times, after which the sludge was considered to be saturated with sewage; it was then allowed to sediment and suspended in water. 1 ml. of this saturated sludge suspension was used in the cups of the Barcroft manometer. By suspension in water and admixture with water and buffer the saturation of the sludge would be somewhat reversed but the results clearly show the inhibiting effect of the preliminary treatment with excess of sewage upon the oxidising activity of the sludge towards further sewage. In Exp. A of Table VII, 1 ml. of sludge was present in the right-hand cup but none in the left, whilst every cup had 0.5 ml. of a Seitz-filtered crude sewage in its side arm, this sewage being tipped into the main contents of the cup at various times after the beginning of the experiment. The figures given are a measure of the extra oxygen absorption due to the added sterile sewage for the first 2 hours after its addition. In Exp. A the figures represent the differences between the corresponding oxygen absorption values for saturated sludge *plus* sterile sewage and saturated sludge *plus* water. It will be seen from these results that the oxygen consumption or the rate of oxidation of the added sewage by the sludge steadily increases with the length of time the saturated sludge undergoes oxidation before the sewage is added. Exp. B, Table VII, shows similar results obtained with another saturated sludge in a different way. In this experiment several Barcroft manometers were used as before but the saturated sludge was placed in both cups, water being tipped into the left-hand cup and sterile sewage into the right-hand cup at the appropriate times. The manometric readings therefore give a direct measure of the oxygen absorption due to the addition of the sewage. As before the figures show that the sewage is not oxidised as rapidly by the saturated sludge as it is by a saturated sludge that has been previously aerated for some time. In this particular experiment it appears that it was necessary to aerate the saturated sludge for about 5 hours before its activity to oxidise further sewage became approximately equal to that of the washed sludge from which the saturated sample was originally prepared. That these results are due to a supersaturation of the sludge with the sewage can be illustrated by the experimental results given in Table VIII. They

Table VIII. *Rates of autoridation of three sludges A, B and C (Exp. 1) and the rates at which these same sludges oxidised added sterile sewage (Exp. 2).*

Sludge A, a suspension of washed activated sludge from the experimental tank; Sludge B, the same sludge after saturation with sewage; Sludge C, sludge B washed several times with water. The oxygen absorption values represent the volumes absorbed in 2 hours, expressed in μ l. at N.T.P. The experiments were made at 22°. The contents of the Barcroft manometers are given in the table, Sl. representing 1 ml. of the appropriate sludge. CS. = 1.5 ml. sterile sewage, B. = buffer, p_H 7.4 (0.5 ml.) and W. = water to make the volume of liquid to 3 ml. CO_2 was absorbed by KOH.

Exp.	Contents of cups		Sludge		
	Left	Right	A	B	C
1	B. + W.	Sl. + B. + W.	34	140	82
2	Sl. + B. + W.	Sl. + B. + CS.	88	30	77

show that sewage had been taken up by the sludge as a result of the preliminary treatment, for the saturated sludge had a much greater rate of autoxidation than either the original sludge or the saturated sludge after several washings with water. Nevertheless, when the rates at which these three sludges oxidise an additional amount of sewage are compared, the saturated sludge is shown to be

much the least efficient. These experiments with saturated sludges appear to emphasise the importance of feeding activated sludge carefully and not with too much sewage and seem clearly to show the value of submitting an activated sludge to periodical aeration in the absence of added sewage, thereby avoiding supersaturation and so allowing the sludge to do its work in the future. Especially is this likely to be so in works practice where the sewage-sludge mixture is not submitted to such complete aeration as it is in the experimental manometer cups.

CONCLUSIONS.

1. The rate of oxidation of sewage increases with rise of temperature from 18° to 37°.
2. Sewage undergoes oxidation readily between p_H 6.0 and 9.0 with a possible optimum at about p_H 7.3. Oxidation to a smaller extent takes place at p_H 5.0 and at p_H 10.0 but at p_H 4.0 it is completely inhibited. The oxidation of washed activated sludges takes place vigorously at all p_H values between 5.0 and 10.0, but none occurs at 12.0. The effect of p_H on the oxidation of sewage in the presence of sludge is the same as for sewage alone.
3. Carbon dioxide is evolved in sewage and sludge oxidations but the ratio CO_2/O_2 is much less than unity; it appears to increase as oxidation proceeds.
4. The harmful effect of supersaturating a sludge with sewage upon the ability of a sludge to oxidise further sewage is clearly indicated and the reversal of this effect by preliminary aeration of the saturation sludge or by its thorough washing is demonstrated.
5. For efficient working of an activated sludge plant therefore the p_H of the system should be kept between 5.0 and 10.0, but preferably between 6.0 and 9.0. In feeding the sludge with sewage care must be taken that the sludge does not become "overloaded" or supersaturated with sewage. Occasional aeration of an activated sludge in the absence of added sewage improves its efficiency for subsequent sewage oxidation.

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XXVI. STUDIES ON THE ACTION OF EMULSIN.

III. SOME SOURCES OF ERROR IN THE POLARIMETRIC EXAMINATION OF ENZYMIC HYDROLYSIS OF β -GLUCOSIDES.

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IN the examination of enzymic hydrolysis of β -glucosides the velocity constant for the hydrolysis and the affinity constant are often determined by following the hydrolysis of the glucoside polarimetrically. This may be done with great accuracy, but we wish to call attention to some sources of error which have not been sufficiently considered in these determinations, and to give material allowing a correction for these errors.

I. The rotation of solutions of glucosides and glucose is dependent on the p_H of the solutions. As the enzymic action of emulsin is stopped by addition of a solution of potassium carbonate to a sample of the glucoside-emulsin solution at p_H 4.4, and the p_H of the resulting mixture is 10.5-10.6 (colorimetrically determined), it is the rotation at the latter p_H which is to be taken into consideration. The difference in rotation at the two different values of p_H is some 3-4 %.

II. Glucose solutions are not stable at p_H 10.5-10.6. Michaelis and Rona [1912] have examined how the rotation of glucose solutions varies with the time at different p_H values and have found, for 10 % glucose solutions, that up to p_H 7.6 the solutions are quite stable, at p_H 10.15 the rotation decreases 0.012° per hour, at p_H 10.65, 0.022° per hour and at p_H 10.82, 0.037° per hour. We have found that for 0.1 *M* glucose solutions (1.8 %) at p_H 10.5-10.6 the decrease of rotation is 0.0033° per hour, i.e. sensibly the same result as Michaelis and Rona. In the examination of enzymic hydrolysis of glucosides samples containing glucose are kept for at least 3 hours at p_H 10.5-10.6 in order to allow the mutarotation to take place, and they may be kept a much longer time, up to some 20 hours, at this p_H before determination of the rotation. Under these circumstances the decrease in rotation of the solution may exceed the limit of error considerably, and a correction must be introduced. We have also examined whether glucose solutions containing emulsin change their rotation with time at p_H 4.4. It has been shown by Bourquelot [1914] and Bourquelot *et al.* [1920] that formation of gentiobiose and cellobiose may take place in solutions of glucose containing emulsin. Bourquelot, however, used glucose solutions much more concentrated than those used in our experiments, but we have thought it useful to examine if any trace of change in rotation may be observable. This was, however, not the case.

III. The standardisation of the emulsin preparation was carried out with salicin as standard glucoside. In a previous paper [Veibel, 1934] we have used the method of Helferich [1931], who quotes a paper of Weidenhagen [1929], published in a journal not very readily obtainable. We have now examined the

original paper of Weidenhagen and have found that the concentration of salicin used by him is not the same as that used by Helferich. Weidenhagen uses a salicin concentration of 3.972 % (in order to be in concordance with the "standard conditions" generally used in the hydrolysis of polysaccharides, *i.e.* a substrate concentration equivalent to a maltose concentration of 2.5 %). Helferich, on the other hand, uses a stock solution of salicin with the concentration 3.972 % and mixes 80 ml. of this solution with 20 ml. emulsin solution, so that the salicin concentration in the mixture is only 3.178 %. The reaction constant divided by the emulsin concentration, *i.e.* sal.f. , is in this way found too high, as the reaction constant increases when the salicin concentration decreases.

In a later paper [1932], dealing with the comparison of the rate of hydrolysis of different glycosides, Helferich seems to have used the salicin concentration indicated by Weidenhagen. He does not mention, however, that this concentration is different from that used in the standardisation of the emulsin preparation.

IV. In experiments dealing with enzymic hydrolysis of glucosides toluene is usually added to the solution in order to prevent the growth of micro-organisms during the experiment. We have examined whether addition of toluene causes any change of the rate of hydrolysis of glucosides and have found the reaction constants to be some 25–35 % higher in experiments with addition of toluene than in experiments without toluene. This means that it is not permissible to compare experiments in which toluene has been added with experiments without toluene, if the extent to which the rate of hydrolysis is altered by the addition of toluene is not stated.

EXPERIMENTAL.

I. Variation of rotation of solutions of glucosides and glucose with p_H .

In Table I are given the values of the rotation of different solutions at p_H 4.4 and at p_H 10.5–10.6. p_H 4.4 is the standard p_H in the hydrolysis experiments. In order to determine the progress of the hydrolysis samples of 5 ml. are

Table I. Values of α at different p_H values (2 dm. tubes).

	Conc.	p_H 4.4	p_H 10.5–10.6
Salicin	5/6 3.972 %	- 4.160°	- 4.280°
β -Methylglucoside	5/6 0.04 <i>M</i>	- 0.440	- 0.455
β -Methylglucoside	5/6 0.10 <i>M</i>	- 1.100	- 1.140
β -Ethylglucoside	5/6 0.04 <i>M</i>	- 0.535	- 0.555
β -Ethylglucoside	5/6 0.10 <i>M</i>	- 1.335	- 1.385
Glucose	5/6 0.05 <i>M</i>	0.795	0.775
Glucose	5/6 0.10 <i>M</i>	1.585	1.530
Glucose	5/6 0.20 <i>M</i>	3.175	3.045

withdrawn and mixed with 1 ml. of 20 % potassium carbonate, the p_H being in this way brought to 10.5–10.6. In order to obtain values at p_H 4.4 comparable with the values at p_H 10.5–10.6 5 ml. are mixed with 1 ml. of water. The difference is in all cases 3–4 %.

II. Variation of rotation of glucose solutions with time.

Solutions of glucose, 0.05 *M*, 0.10 *M* and 0.20 *M* were prepared and were kept 24 hours to allow the mutarotation to take place. Samples of 5 ml. were then mixed with 1 ml. of 20 % potassium carbonate and kept different times before determination of the rotation. Table II gives the results.

Table II. *Variation with time of rotation of glucose solutions at p_H 10.5–10.6.*

Time after addition of K_2CO_3 (hours)	α	$\Delta\alpha$	$\Delta\alpha/\text{hour}$	$\Delta\alpha/\text{hour, } 0.1\text{ }M$
Glucose 0.05 M				
0	0.775°	—	—	—
3.25	0.770	0.005°	0.0015°	0.0030°
28.5	0.720	0.055	0.0019	0.0038
51.75	0.665	0.110	0.0021	0.0042
Glucose 0.10 M				
0	1.530	—	—	—
3.5	1.520	0.010	0.0029	0.0029
28.75	1.450	0.080	0.0028	0.0028
52.0	1.360	0.170	0.0033	0.0033
Glucose 0.20 M				
0	3.045	—	—	—
3.75	3.020	0.025	0.0067	0.0034
29.0	2.880	0.165	0.0057	0.0028
52.25	2.655	0.390	0.0075	0.0038
Mean value				0.0033°

Table III shows that the presence of emulsin causes no change in the rotation of glucose solutions up to 0.20 M and 54 hours.

Table III. *Rotation of glucose solutions containing emulsin.*

Time after addition of emulsin hours	0.05 M α	0.10 M α	0.20 M α
0	0.535°	1.290	2.790
30	0.535	1.290	2.795
54	0.535	1.290	2.795

The rotation of the emulsin control was -0.240° during the whole of the experiment.

III. *Standardisation of the emulsin preparation.*

As indicated above the rotation of the 3.972 % salicin solution, when 5 ml. are mixed with 1 ml. 20 % potassium carbonate, is -4.280° , and when they are mixed with 1 ml. water -4.160° , which is the value calculated from the $[\alpha]_D$ of salicin, -62.8° . The rotation of the solution after complete hydrolysis is at p_H 10.5–10.6 $+2.150^\circ$, at p_H 4.4 $+2.190^\circ$. The total change of rotation is thus at p_H 10.5–10.6 6.430° , at p_H 4.4 6.350° .

If no regard is paid to the influence of p_H on the rotation, so that the value at time 0 is calculated to be -4.160° , it may be seen that the reaction constant found is too low. This is to some degree counterbalanced since the value for the total change of rotation is also calculated too low. In Fig. 1 we give the results of two experiments with different emulsin concentrations, calculated in both ways.

Table IV gives the times required for 50 % hydrolysis, the corresponding reaction constants (unimolecular, calculated with logarithms to base 10 and the minute as unit of time), sal.f., which is k_{30°/e (e = g. emulsin in 50 ml. reaction mixture) and β -glucosidase values = sal.f./log 2.

The emulsin preparation is the same as that used in the previous paper [Veibel, 1934]. Its sal.f. was there determined by the method of Helferich [1931] and was found to 0.045. We now find that sal.f., if corrected for the influence of p_H , = 0.043; if not corrected, = 0.041.

Table IV. *Corrected and uncorrected values for salicin.*

	50% hydrolysis		$k_{50\%}$		sal.f.		β -glucosidase values	
	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.
$e = 0.0691$	103	108 min.	29.2	$27.9 \cdot 10^{-4}$	0.042	0.040	0.140	0.134
$e = 0.1382$	50	53 min.	60.2	$56.8 \cdot 10^{-4}$	0.044	0.041	0.145	0.136

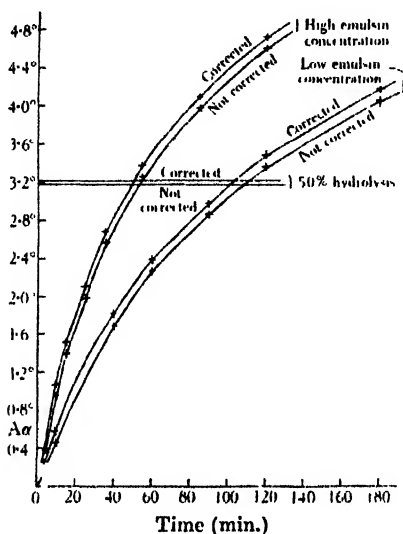


Fig. 1.

IV. *The influence of addition of toluene.*

From a stock solution of methylglucoside in acetate buffer ($p_H = 4.4$ and $0.1 M$) and a stock solution of emulsin in water two measuring flasks of 50 ml. were filled at 30° . Each flask contained 0.2213 g. emulsin and was $0.04 M$ with regard to methylglucoside. To one of the flasks 1 ml. of toluene was added. Samples were withdrawn as usual. Tables V and VI give the results.

In the experiment without toluene k/e (sal.f.) = $2.7 \cdot 10^{-2}$, whereas the corresponding value in the experiment with toluene is $3.6 \cdot 10^{-2}$. For ethyl- β -glucoside a corresponding difference was found: k/e (sal.f.) = $5.3 \cdot 10^{-2}$ without toluene, $7.6 \cdot 10^{-2}$ with toluene. We have not sought for an explanation of this effect of the addition of toluene. Our emulsin solutions are made by mixing 1 g. of emulsin with 100 ml. of water. After some time the solution is filtered. Some

Table V. *β -Methylglucoside. Hydrolysis without addition of toluene.*

Time min.	Samples kept at p_H 10.5 hours	α	$\alpha_{corr.}$	α	$c - \alpha$	$k \cdot 10^4$
0	4.0	-0.810°	-0.810°	—	1.075°	—
120	3.5	-0.730	-0.730	0.080°	0.995	2.80
240	20.5	-0.680	-0.675	0.135	0.940	2.43
360	19.0	-0.615	-0.610	0.200	0.875	2.48
640	15.0	-0.460	-0.455	0.355	0.720	2.72
1440	5.5	-0.220	-0.215	0.595	0.480	2.43
Mean value						2.57

Table VI. β -Methylglucoside. Hydrolysis with addition of toluene.

Time min.	Samples kept at p_H 10.5 hours	α	α_{corr}	x	$c - x$	$k \cdot 10^4$
0	4.0	- 0.810 ^o	- 0.810 ^o	—	1.075 ^o	—
120	3.75	- 0.715	- 0.715	0.095 ^o	0.980	3.35
240	20.75	- 0.635	- 0.630	0.180	0.895	3.32
360	19.25	- 0.545	- 0.540	0.270	0.805	3.49
640	15.25	- 0.380	- 0.370	0.440	0.635	3.58
1440	5.75	- 0.095	- 0.090	0.720	0.355	3.34
2940	3.25	+ 0.095	+ 0.100	0.910	0.165	(2.77)
4320	4.25	+ 0.215	+ 0.220	1.030	0.045	—
5760	4.0	+ 0.235	+ 0.240	(1.050	0.025	(—)
Mean value						3.42

The rotation of the emulsin control was in all cases but the last - 0.355°. After 5760 min. the rotation was - 0.340°. That is to say that the equilibrium point has been reached in about 4320 min.

0.7–0.8 g. is dissolved in this way. The filtrate is quite clear. When the emulsin solution is added to the buffer solution a slight flocculation usually takes place. It may be that some active substance is precipitated in this way and that it is then brought into solution by the addition of toluene. In a recent paper Willstätter and Rohdewald [1934] have called attention to the fact that toluene + water may be a different dispersion-medium from water alone. They state that the activity of lipase solutions decreases when toluene is added. They do not mention, however, if they have ever found an activation of ferments by toluene such as we have found here for emulsin.

SUMMARY.

Attention is called to some sources of error in the polarimetric examination of enzymic hydrolysis of glucosides, namely:

1. Solutions of glucose and glucosides have not the same rotation at p_H 10.5–10.6 as at p_H 4.4. The difference is some 3–4 %.
2. Glucose solutions kept at p_H 10.5–10.6 are not stable. Their rotation decreases with time; for 0.1 M solutions 0.0033° per hour.
3. The salicin concentration used by Helferich [1931] for standardising emulsin preparations is not the same as that indicated by Weidenhagen [1929].
4. When toluene is added to solutions of β -glucosides, used in the examination of the rate of enzymic hydrolysis, the reaction constants are found to be some 25–35 % higher than when no toluene is added.

Our thanks are due to the Carlsberg Foundation for a grant which enabled one of us (F. E.) to take part in this work.

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XXVII. THE DETERMINATION OF STARCH IN PLANT TISSUE, WITH PARTICULAR REFERENCE TO THE APPLE FRUIT.

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THE quantitative determination of starch in plant tissues remains a difficult matter in spite of the numerous analytical methods described. Although two recent procedures [Denny, 1934; Sullivan, 1935], based on preliminary extraction of the starch in boiling concentrated CaCl_2 solution [after von Fellenberg, 1916], have attained a high degree of selectivity for true starch, both methods are laborious. In the present paper a relatively simple procedure is described which appears to be strictly specific for starch and to give unusually exact values. With certain precautions its application to tissues other than the apple should present little difficulty.

The starting material for starch determination, as in most methods, is the residue left after thorough extraction of the finely-divided tissue with hot 70–80 % alcohol. The particular advantages of the method are due to the two following innovations: (1) The tissue residue is subjected to a treatment which converts native starch into "soluble" starch; (2) use is made of β -malt-amylase (from ungerminated barley) as a selective hydrolysing agent.

(1) The "solubilising" treatment is based on the observation [Small, 1919] that hot dilute alcoholic HCl converts starch into a highly soluble form, with minimum production of reducing products.

A suspension of the material in boiling 95 % alcohol is treated with 1/100 vol. of conc. HCl and vigorous boiling is continued for 12 min. under a reflux condenser. After rapid cooling, the residue is drained on a suction filter and washed by gradual addition of an equal volume of 95 % alcohol. In practice, weights of apple residue up to 1.5 g. (\equiv about 50 g. fresh weight) were treated with 100 ml. 95 % alcohol *plus* 1 ml. HCl; for larger amounts proportionately larger volumes were used to ensure adequate mixing by the boiling of the alcohol.¹

After this standard solubilising treatment starch can be removed quantitatively from the residue by hot water extraction. In addition, solubilisation facilitates rapid action of amylase on starch.

(2) The second innovation, namely the use of β -malt-amylase, is largely responsible for the improved selectivity. Highly active preparations are readily obtainable (Appendix I) which contain probably no other carbohydrase.

A further advantage is that the reducing products of the action of this enzyme consist almost exclusively of maltose, a characteristic which is the basis of its use in the standard method of preparing maltose [Harding, 1923; *cf.* Hanes, 1935].

The degradation of starch by β -amylase proceeds to a definite "hydrolysis limit" which is independent of enzyme concentration and is only slightly

¹ Note added January 8th, 1936. Attention is called to the recent paper by Niemann *et al.* [1935, *Plant Physiol.* 10, 579] in which a preliminary solubilising treatment with alcoholic HNO_3 is described.

influenced by variation in the method of solubilising the substrate.¹ The value is remarkably constant when the latter is controlled (*cf.* Table I).

In order to convert into its equivalent of starch an observed increase in reducing power resulting from the action of the enzyme on a tissue extract, a knowledge of the hydrolysis limit for the particular variety of starch is necessary. Starches from potato and apple after the standard solubilising treatment are found to give almost identical values, the increase in reducing power corresponding to 0.603 and 0.600 mg. maltose, respectively, per mg. starch. It is probable from van Klinkenberg's [1932] results that similar values hold for wheat, arrow-root and buckwheat starches [*cf.* Hanes, 1935]. However, since some starches may differ in this respect, it is advisable to make fresh determinations for each individual variety.

EXPERIMENTAL.

1. *Technique of determination.*

The same procedure was followed in determining maltose production by the action of β -amylase on solutions of purified apple starch (control starches) and on filtrates from apple tissue.

Values for increase in reducing power are based on differences in reduction values of parallel digests with active and "killed" enzyme (inactivated by holding at 90° for 10 min.), the conditions and duration of reaction being such as to ensure attainment of the hydrolysis limit.

Digests were maintained at the optimum acidity by the addition of acetate buffer [Cole, 1933] of p_H 4.63 (final concentration $N/70$ to $N/90$), at a temperature of 25°, the final starch concentration not exceeding 0.25%. Pipettes were plugged with cotton-wool to exclude traces of saliva, and toluene was added as a preservative.

It is convenient to use a concentration of enzyme considerably in excess of that actually required to accomplish the hydrolysis within the selected reaction period. (This avoids the need for more than occasional examination of its activity, which for the present purpose is best done by ascertaining that no maltose is produced during the final quarter of the period.) Analysis of digests in the subsequent experiments was done after approximately 42 hours' reaction. In general, digests were of 28 ml. final volume, comprising 25 ml. substrate (control starch or tissue filtrate), 2 ml. $N/5$ acetate buffer and 1 ml. stock β -amylase solution (Appendix I), but in some cases 2 ml. enzyme were used.

In general an alkaline ferricyanide reagent² [Hanes, 1929] was used for maltose determinations on 5 ml. (occasionally 3 ml.) digest samples, but in addition a copper reagent, Reagent 60 of Shaffer and Somogyi [1933], was used for comparison, after standardisation against maltose.

¹ Maltose production ranging from 60 to 67% theoretical has been reported for this enzyme by different investigators. Part of the variability arises from the custom of expressing the hydrolysis limit in terms of final reduction value without correction for initial reducing power of starch and enzyme. In addition to maltose a non-reducing dextrin (erythrogranulose) is produced. These points are discussed elsewhere [Hanes, 1935].

² With certain batches of potassium ferricyanide (supplied as A.R. grade) deviations from the normal linear standardisation curve for maltose have been observed, but after purification by twice recrystallising the salt from solutions saturated at 50° the discrepancy has disappeared. This purification has accordingly been adopted as a routine procedure. With this reagent 1 ml. $N/100$ thiosulphate \equiv 0.413 mg. anhydrous maltose.

2. Determination of hydrolysis limit on control starches.

Substrate solutions were prepared in a standard manner from the five specimens of soluble apple starch described in Appendix II. A weighed amount of starch, suspended in cold water, is washed into a larger volume of boiling water. The suspension is boiled 3 min., held at 100° on a steam-bath for 20 min. longer, then cooled and brought to volume. Concentrations are given in terms of anhydrous starch.

In a number of experiments the effect of varying enzyme concentrations was studied. The results showed that, provided sufficient enzyme is added, it may be widely varied without affecting the final value for maltose production. For example, in a series of digests (final volume 29 ml., containing 4.11 mg. control starch/5 ml.) the additions of enzyme were 0.1, 0.2, 0.3 and 0.5 ml., respectively, of stock enzyme. The final increases in reducing power indicated the production of 2.42, 2.46, 2.45 and 2.46 mg. maltose/5 ml.

In the experiments summarised in Table I the enzyme concentration was most commonly double the maximum amount above, but in some cases four times that amount.

The hydrolysis limit has been determined both with dry enzyme and, as recommended for routine use, with preparations stored in solution.

Table I. *Observed hydrolysis limits for soluble apple starch.*

S, mg. anhydrous starch/5 ml. digest.						
M, increase in reducing power as mg. maltose/5 ml.						
M/S, mg. maltose produced from 1 mg. starch.						
1. Using stock β -amylase stored in solution						
Starch A	S	2.045	2.045*	4.09	4.09*	10.21
	M	1.25	1.24	2.49	2.46	6.10
	M/S	0.611	0.606	0.600	0.601	0.597
Starch B	S	0.80	3.99	3.99*	—	—
	M	0.47	2.385	2.38	—	—
	M/S	0.589	0.598	0.597	—	—
Starch C ₁	S	2.015	4.03	4.03*	6.045	—
	M	1.29	2.415	2.42	3.06	—
	M/S	0.612	0.600	0.601	0.606	—
Starch C ₂	S	2.06	2.06*	4.12	4.12*	6.18
	M	1.26	1.22	2.46	2.47	3.72
	M/S	0.611	0.592	0.597	0.599	0.6015
Starch D	S	1.255	4.11	4.11	4.11	6.18
	M	0.76	2.46	2.45	2.46	3.72
	M/S	0.606	0.598	0.596	0.598	0.601
2. Using dry β -amylase preparation						
Starch B	S	1.96	3.26	3.26*	3.91	—
	M	1.15	1.945	1.95	2.36	—
	M/S	0.588	0.596	0.599	0.600	—

* In these cases reduction values were by the copper reagent, all others by ferricyanide reagent.

The *M/S* values are closely reproducible both with varying starch concentrations and with different starch specimens. The average value of maltose production by the completed action of the enzyme in the 26 cases given is 0.600 mg. maltose/mg. starch, the extremes being 0.588 and 0.612. The different specimens were prepared from fruit picked throughout the growing season and the average sizes of the starch grains in the different cases ranged from 5.0

to 9.2μ in diameter (C_1 and C_2 , respectively). It is apparent that neither of these factors influences the hydrolysis limit by β -amylase.

Moreover, the good agreement observed by the use of the ferricyanide and the copper reagents is convincing evidence of the homogeneity of the reducing material produced, since these reagents were selected as giving widely different reducing ratios for glucose and maltose.

3. *Extraction of starch from tissue residues.*

Starch determinations were made usually on 1 g. portions of alcohol-extracted residue (≈ 22 – 33 g. fresh apple tissue). The residue is subjected to the standard solubilising treatment (p. 168), after which the alcohol is allowed to evaporate. The product, suspended in about 110 ml. water, is boiled 3 min. and held at 100° for a further 20 min. It is then cooled and diluted to 200 ml. The suspension is filtered with suction (ordinary non-hardened paper) and 25 ml. portions of the filtrate are used as substrate in digests with active and "killed" enzyme (cf. p. 169).

This technique, adopted as a routine for investigating apple tissue, would require appropriate modification for other tissue with different starch content.

That the whole of the starch contained in solubilised residues was dissolved by treatment with hot water was shown as follows. Residues were extracted with 3 and 4 successive lots of boiling water and the enzyme allowed to act both on portions of the combined filtrates, and on suspensions of the extracted residues. In all cases the whole of the starch was present in the filtrates, there being negligible amounts of hydrolysable material left in the extracted residues.

The validity of the standard technique for preparing filtrates is attested by the following experiment.

Four portions of a solubilised residue were extracted as follows:

1. 1 g. residue, suspended in water, heated in the standard way and cooled, was made up to 100 ml. before filtration.
2. 1 g. residue treated as in 1, but diluted to 200 ml. before filtration (*i.e.* the standard extraction).
3. 1 g. residue extracted with four successive portions of water, the filtrates being made to 200 ml.
4. 0.486 g. residue subjected first to standard heating in 75 ml. water, then extracted 4 hours in a Soxhlet apparatus. Final volume 71 ml.

The results of β -amylase action are given in Table II.

Table II.

Extract	Tissue equivalent to 5 ml. final digest		Maltose production* mg./5 ml.	\equiv starch† mg.	Starch per g. mg.
	Fresh wt. mg.	Residue mg.			
1	392	17.9	1.825 – 0.605	2.04	5.19*
2	392	17.9	1.83 – 0.62	2.015	5.14
3	392	17.9	1.835 – 0.635	2.005	5.11
4	267	12.2	1.525 – 0.795	1.38	5.16

* Reducing values of digests with active and "killed" enzyme are given.

† Obtained by dividing "maltose production" by 0.600 (the hydrolysis limit).

There is good agreement in the values obtained with the different extraction methods. The slightly higher value for Extract 1 may be due to the volumetric error (from the presence of solids) being significant when the suspension was diluted to only 100 ml. before filtration.

4. *Recovery of added starch.*

Starch added to residues before the solubilising treatment is determined quantitatively by the method. The results are given of an experiment carried out with virtually starch-free apple tissue.

Five 1 g. portions of residue ($\equiv 31.5$ g. fresh weight) were used. To four of these were added amounts of native apple starch as follows—89.1, 222.9, 357.1 and 444.8 mg. The residues were then subjected to the standard solubilising and extraction procedure.

The observations are given in Table III.

Table III.

5 ml. digest equivalent to

mg. fresh weight	mg. added starch	Maltose production mg./5 ml.	\equiv starch mg.	Increase mg.	% recovery
704	—	0.675 - 0.62	0.09	—	—
704	1.99	1.88 - 0.64	2.07	1.98	99.5
704	4.97	3.73 - 0.705	5.04	4.96	99.8
704	7.96	5.71 - 0.75	8.23	8.16	102.4
704	9.93	6.87 - 0.77	10.17	10.08	101.5

The results illustrate both the accuracy with which added starch is recovered and the specificity of the method since for the first residue (without added starch) a value approaching zero was obtained.

Results (not given under experimental).

Analysis of apples during storage. Analyses were made on a series of residues (from an experiment conducted by Dr F. Kidd) for which determinations of material hydrolysed by takadiastase were available.¹ The series represents a succession of samples of young apples (Bramley's Seedling) removed from a population stored at 15° from the time of picking.

By the 10th day slices treated with 1-KI appeared starch-free macroscopically, but under the microscope showed traces of starch. By the 190th day the tissues were virtually starch-free, only a few minute grains being seen in a large number of slides examined.

Table IV.

Days at 15°	5 ml. digest \equiv mg. fresh wt.	Maltose production mg./5 ml.	\equiv starch mg./5 ml.	mg. "starch" per g. fresh weight		Difference mg./g.
				β -Amylase method	Taka-diastase method	
0.5	486	2.36 - 0.72	2.74	5.64	9.20	3.56
1	500	2.02 - 0.695	2.21	4.42	7.93	3.51
2	510	1.99 - 0.74	2.08	4.08	7.58	3.50
3	552	1.545 - 0.67	1.455	2.63	5.59	2.96
4	559	1.37 - 0.73	1.07	1.92	4.09	2.17
6	582	0.99 - 0.775	0.36	0.62	2.41	1.79
8	623	0.795 - 0.695	0.17	0.27	1.60	1.33
10	625	0.73 - 0.69	0.07	0.11	1.55	1.44
190	686	0.64 - 0.61	0.05	0.07	1.37	1.30

¹ I am indebted to Dr Kidd for allowing me to use the takadiastase data. In these determinations (which were made by Miss D. G. Griffiths and Miss N. A. Potter) an excess of takadiastase was allowed to act to completion on the unfractionated residues after suspension in water and heating at 100° for 30 min. The resulting reducing material was considered, for convenience in expressing the results, to be glucose produced from starch hydrolysis, and was converted into a starch value by a factor obtained from the action of the enzyme on starch paste.

Observations on filtrates from these samples are given in Table IV. Throughout the series 5 ml. digest correspond to 22.3 mg. residue, and the equivalent fresh weights are given in the table.

The values obtained by the two methods are plotted in Fig. 1, upper graph, and the differences in the lower graph.

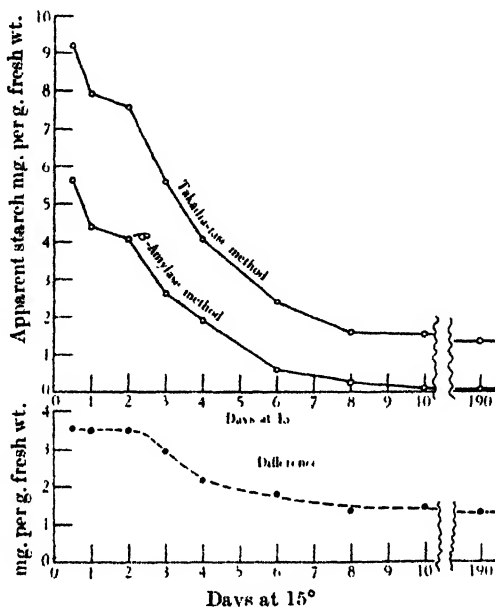


Fig. 1. Upper: determinations by β -amylase and takadiastase methods on apples stored at 15°. Lower: takadiastase value - β -amylase value.

By the 10th day the values by the β -amylase method, initially considerably smaller than the takadiastase values, fall to a low level approaching zero. The takadiastase values fall to about 1.5 mg. per g. and remain at about this level during subsequent storage although the true starch content falls to zero.

The lower graph suggests that two distinct classes of material are hydrolysed by takadiastase but not by β -amylase, one of which disappears during storage and so is presumably metabolised, whilst the other persists. That two non-starch fractions hydrolysable by takadiastase are present was suggested by the

Table V.

	5 ml. digest mg. fresh weight	Maltose production mg./5 ml.	≡ starch mg./5 ml.	mg. "starch" per g. fresh weight	
				β -Amylase method	Takadiastase method
Onion	2230	1.06 - 0.99	0.11	0.05	—
	1115	0.72 - 0.70	0.035	0.03	—
Wheat leaf	370	0.60 - 0.575	0.04	0.1	7.1*
	351	0.52 - 0.48	0.08	0.2	6.9
Wheat sheath	342	0.53 - 0.48	0.08	0.25	12.1
	418	0.595 - 0.54	0.09	0.2	10.0

* These samples were kindly supplied by Dr H. R. Barnell, School of Agriculture, Cambridge, who had previously made takadiastase determinations on the same residues and concluded on other grounds that the material hydrolysed was not starch.

further observation that about 1/3 of the "extra starch" by takadiastase was not extractable by hot water, whilst 2/3 was present in the filtrates, when a portion of the 0.5 day residue was so fractionated.

Analysis of other tissues. The selectivity of the method was further confirmed in an examination of two types of tissue in which starch is either completely absent (the onion) or is present only in traces in stomata (leaves and leaf sheaths of wheat). The observations are given in Table V.

These examples demonstrate further the selectivity of the method.

SUMMARY.

A method for the determination of starch in plant tissues is described which appears to combine the advantages of selectivity, accuracy and relative technical simplicity.

After preliminary extraction of the tissue with 70–80 % alcohol the residue is boiled in dilute alcoholic hydrochloric acid to convert native starch into soluble starch. The starch is then completely extractable in hot water. Soluble starch in the extract is next selectively hydrolysed by β -malt-amylase (from ungerminated barley). Various advantages conferred by the use of this enzyme are discussed, in particular its specificity as a starch "reagent", the production of maltose as the almost exclusive reducing product and the existence of a definite hydrolysis limit.

Before applying the method to apple tissue a number of specimens of apple starch were isolated in the form of starch grains. Throughout the season, apple starch is hydrolysed to the same extent by β -amylase, the increase in reducing power being equivalent to the production of 0.600 mg. maltose per mg. starch. This value agrees with that for potato starch under similar treatment.

The method is illustrated by a number of analyses on apple and other tissues.

I wish to acknowledge the careful technical assistance of Mr Eric Whitmore.

APPENDIX I.

Preparation of enzyme.

Ungerminated barley of good malting quality (preferably pale in colour) is ground to a fine flour. Metal-free water (from an all pyrex still) is used in the preparation.

200 g. of the flour are extracted with 750 ml. 50 % ethyl alcohol for 2 hours with stirring. After centrifuging, the residue is re-extracted (400 ml. 50 % alcohol; 0.5 hour) and again centrifuged. The supernatant liquors are combined and after filtration the enzyme is precipitated by increasing the alcohol concentration to 80 %.

The precipitate, after removal on the centrifuge, is well drained on a suction filter and suspended in 450 ml. water. Toluene is added and the solution is stored in the dark at 1°. (It may require filtration after several days to remove a flocculent precipitate.)

With reasonable precautions against heavy metal contamination (from dust and unclean pipettes) such preparations may be used for several months; inactivations of the order of 20 % are to be expected after 3–4 months' storage.

For routine purposes these solutions are preferable to dry preparations since the latter, although more stable, are extremely difficult to dissolve.

APPENDIX II.

Preparation of control starches from apples.

Five specimens of apple starch (in the form of starch grains) were prepared from fruit (Worcester Pearmain) picked at different stages of maturity from July 11th to Sept. 2nd.

Apples were frozen at -20° soon after picking; the peel was removed and the flesh ground to a powder while still frozen. The powder was suspended in a large volume of water at 1° with stirring. (For Specimens A and B the powder was dehydrated and then boiled in 95% alcohol before suspension in water; for Specimen D, 10 mg. KCN per litre were added to the water.) The suspension was then filtered through fine silk which allowed the finer particles, including starch grains, to pass. The washing of the residue was repeated several times.

Isolation of the starch from the filtrates was now carried out on the centrifuge. By repeated fractional sedimentation in water and 70% alcohol a fine grey sludge was ultimately removed. The pure product was then washed in 95% alcohol, absolute alcohol and ether.

Specimens C_1 and C_2 are two fractions of a single preparation, fine-grained and large-grained, respectively. The separation was accomplished by 24 hours' sedimentation of the partially purified product (about 12 g.) in 6 litres of water. The finer grains, removed from the supernatant liquor on the centrifuge, and the larger grains, which had settled, were then worked up separately.

The range of diameter of starch grain and the average diameter (from 100 random measurements) for the different specimens were as follows (in μ): A, 2-9, 5.5; B, 2-14, 6.5; C_1 , 3-7, 5.0; C_2 , 5-13, 9.2; D, 3-13, 7.1.

The control starches were prepared from these specimens of native apple starch by subjecting portions (2-5 g.) to the standard solubilising treatment. No loss of dry matter occurred during this process.

Determinations of moisture were made by drying to constant weight at 110° in a high vacuum in a vessel with one limb containing P_2O_5 at room temperature.

The solubilised starches had a small reducing power which ranged from 0.7 to 1.4% of the reducing power of maltose.

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XXVIII. CEREALS AND RICKETS.

VI. THE COMPARATIVE RICKETS-PRODUCING PROPERTIES OF DIFFERENT CEREALS.¹

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UP to 1921 little attention had been given to the variation in the rickets-producing capacity of different cereals or cereal products. E. Mellanby [1921], working with dogs, reported a wide variation in this respect between cereals, particularly between oatmeal and white flour. Later [1925] he reported additional data in support of his earlier findings and offered a hypothesis to explain the observed differences. At this time our experience with rats and dogs had impressed us with the similarity of cereals in the production of mild rickets and we reported briefly [Steenbeck *et al.* 1927] these preliminary observations. Later Green and E. Mellanby [1928] reported a confirmation of Mellanby's earlier conclusions; this time also employing the rat as the experimental animal.

So far as we are aware, E. Mellanby and co-workers, as quoted above, M. Mellanby [1928; 1929; 1930], Holst [1927], Mirvish [1929; 1930], Mirvish and Bosman [1929], Christiansen [1934], and Steenbeck *et al.* [1927; 1930] have compared various cereals or cereal products for calcification of bone or teeth. In contrasting oatmeal with other cereals and their by-products, E. Mellanby [1925] referring to conditions in England states, "Apart from extreme malnutrition, however, it would appear not improbable that in this country, where the average diet is either deficient in or contains a border-line quantity of anti-rachitic vitamin and calcium, and where sunshine is negligible, the ingestion of oatmeal during pregnancy and lactation of women, and in growing children, does much harm."

If Mellanby's contentions be true, then in view of the prominent place which cereals and their by-products occupy in both human and livestock nutrition, more attention should be given to the comparative study of the specific rickets-producing properties of various cereals in spite of the availability of efficient corrective measures. In this paper we are reporting three series of experiments using the low calcium diets of E. Mellanby, which serve as a check on our earlier conclusions.

EXPERIMENTAL.

The experiments of Series I were carried out with four cereals, *viz.* yellow maize, rolled oats,³ whole wheat and patent flour. To eliminate variations in consumption of ration as a determining factor in the outcome of the experiment we equalised the food intake. In each case the consumption was so adjusted that it

¹ Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison, Wisconsin. The data here recorded were taken from a thesis submitted by one of us (B. H. T.) in 1929 in partial fulfilment of the requirements for the Ph.D. degree.

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³ A form of oatmeal representing at least 95 % of all oatmeal used for human consumption in the U.S.A.

was always limited by the least amount consumed by any rat in the various groups, except when that rat showed obvious abnormalities due to other causes than those attributable to the ration. We have used this technique of "equalised consumption" for the last twelve years and consider it indispensable under certain conditions. Mitchell and Beadles [1929] have adopted a similar technique as "paired feeding".

When the nature of the results of this first series of experiments was becoming evident, Series II was started with the same cereals except for the addition of polished rice. This series was run to determine whether the obvious variations from Green and E. Mellanby's [1928] results might not be explained by the fact that they did not secure accurate control of consumption. Green and Mellanby state that they fed 7.5 g. of the cereal and 2.5 g. of the supplement daily, but at the same time make it clear that in the early part of their experiments the animals did not always consume the amount offered. It also is very obvious that with animals of the weight and size used by them it would have been exceedingly difficult, if not impossible, to secure the 10 g. daily consumption. Neither do Green and Mellanby state how much was left unconsumed, and therefore the reader is unable to come to any definite conclusions, nor is he in position to evaluate the variations. To determine the variations which might have been brought about by their technique, we practised *ad libitum* feeding in this series but at the same time kept careful records of the amounts consumed from day to day.

Series III was started, again using the technique of "equalised consumption". Here also five cereals were used, the same as in Series II. Except for the one additional cereal, *viz.* polished rice, this series therefore represents a duplication of the first one.

All the rats used for the experiments were of a piebald closely inbred stock of the same ancestry as that which has been used in our laboratory for the last twenty years. They were raised on our stock ration [Steenbock, 1923] slightly modified to the extent that 5% of butter fat was mixed in with the grain ration to provide a sufficient excess of vitamin A so as to make possible continuous breeding without rest periods after weaning of the young. With this addition of butter fat we have never observed deficiencies of vitamin A in the stock ration. Nor have we ever noticed that the addition of this amount of butter fat introduces sufficient vitamin D appreciably to affect the storage reserves of the young.¹

Six animals were put on each cereal except in Series I where only four were used. In order to equalise as much as possible the effect of litter variations in the development of bone, each group of rats kept on a cereal had apportioned to it a representative from each of the litters used. Furthermore, an attempt was made to secure the same number of the two sexes in each group, because it is a well-known fact, first observed by Hammett [1925], that females uniformly have a slightly higher ash content in their bones than males. In Series I there were three females to one male for each cereal; in Series II, 3 females to 3 males, and in Series III, 2 females to 4 males. In all cases only litters approximating to 60 g. in weight per rat at an age of approximately 25 days were used. The actual weights taking the series in order I to III were as follows: 58.9, 58.2 and 58.4 g. with ranges in each series of 55 to 63, 54 to 62 and 55 to 64 g., respectively. The average ages were 24, 23.9 and 26.7 days for Series I, II and III respectively, with ranges in each of 23 to 25, 22 to 26 and 26 to 29 days, respectively. The rats were confined individually in rectangular cages measuring 10 × 10 × 20 in. Each cage was provided with an elevated false screen bottom of large mesh.

¹ The results with this ration can be markedly improved by the supplemental feeding of "greens" *ad libitum* two to three times a week.

In the experiments already published on rickets we have depended for the most part upon the use of the 3143 ration of McCollum, Simmonds, Shipley and Park [1922, 3] or the 2965 ration of Steenbock and Black [1925] for the production of rickets. In the present series of experiments we used the same ration formulae as those used by Green and E. Mellanby [1928]. The cereals were finely ground in a ball mill. It has been our general experience that cereals of a hard and flinty nature, such as rice and maize, tend to reduce consumption in young rats undoubtedly owing to difference in their physical texture, because we have not experienced this when they were finely ground.

All our rations as in the experiments of Green and E. Mellanby [1928] were fed uncooked. As supplements to the cereals we used a basal mixture of marmite 32 g., caseinogen 24 g., cabbage 25 g., lemon juice 32 g. and sodium chloride 12 g. One part of this was used with 3 parts of cereal. The marmite was furnished in the form of "Vegex". We were assured by the manufacturers that this product was identical with marmite except for the fact that it contained somewhat larger quantities of sodium chloride. The caseinogen was furnished in the form of commercial "casein", which we extracted repeatedly with dilute hydrochloric acid to reduce its content of minerals, particularly calcium. The use of the purified caseinogen we considered especially important in comparing cereals for rickets production, because E. Mellanby [1925] and Green and E. Mellanby [1928] emphasised repeatedly that the differences in the anticlastic properties of the various cereals are particularly evident on a low intake of calcium in the absence of vitamin D. The dried cabbage represented shredded cabbage, from heads which had been cored and freed almost entirely from green leaves, dried at a temperature of 55° overnight. After drying it was finely ground for later mixing with the other constituents.

The various supplements, namely the marmite ("Vegex"), caseinogen, cabbage, lemon juice and in addition sodium chloride, were mixed in small quantities at intervals of 3 or 4 days. This intermittent mixing of the supplement, though not the most desirable procedure, was followed because the marmite was of salve-like consistency and possibly could have lost some of its original nutritional value by drying and exposing to the atmosphere. This mixture was weighed out in portions equal in number to the various cereals fed, and each portion remixed with a cereal in the proportion of 1 supplement to 3 cereal. Equal portions of the final mixtures were then weighed out daily for each rat in the various cereal groups. The daily portions were fed in small tin cups. At the close of the experiments, the accumulated spillage observed in the bottom of the pans was found to be negligible.

In making up the rations as above outlined, we made every attempt to duplicate the Green and E. Mellanby [1928] technique from their description. It must, of course, be emphasised that it was impossible to duplicate their conditions exactly. In the first place, cereal grains and their products are not constant in composition. In the second place, it is very obvious that the cabbage and lemon juice as well as caseinogen and marmite, were somewhat different in composition unless mere chance should have made them the same. It is, furthermore, possible that the cabbage may in one or the other case have contained some vitamin D. We sought to remove this possibility in our experiments by discarding the outside green leaves of the heads. As to whether this was done by Green and E. Mellanby we were unable to determine from their paper. The cereals in our experiments, with the exception of the yellow maize in Series I, were taken from one source. The supplements in Series II and III were the same but of different origin from those used in Series I. The lemon juice, being

extracted from fresh lemons every third or fourth day, was, of course, different in each series.

The animals were weighed weekly, at which times detailed notes were made on their physiological condition. These included not only the observed tendencies to reduced food consumption, but also their general activity and incidence of ophthalmia. It is to be noted that in none of these experiments were there ever observed the slightest signs of vitamin A deficiency. All the experiments were terminated after five weeks. The animals were anaesthetised with ether and bled from their carotids by the technique of Bethke *et al.* [1923] for pooled samples of blood. The bloods were kept in a refrigerator overnight, then centrifuged and the sera used for calcium and inorganic phosphorus determinations according to the techniques of the Clark-Collip [1925] modification of the Tisdall method and the Briggs [1922] modification of the Bell-Doisy method respectively. The wrist bones were dissected free and then preserved in 10 % formalin for later examination of the widening of the epiphyseal line as occurs in rickets. For this we used the technique developed in the Johns Hopkins laboratories [McCollum, Simmonds, Shipley and Park, 1922, 3] except for the fact that our observations were made only in gross without the use of a microscope. The ribs were also preserved in 10 % formalin for later macroscopic comparison as to degree of involvement of the costochondral junctions. The freshly dissected and cleaned femora were collectively and continuously extracted with alcohol for a period of five days in a Soxhlet apparatus. They were then dried, weighed and ashed in an electric muffle furnace for determination of their ash content. We used this technique of ash analysis after Dibbelt [1909] had pointed out that variations in the lipid content of bones brought about big variations in the percentage of ash unless such lipoids were first removed.

Food consumption.

The average data on consumption of rations are shown in Table I. We consider these data as accurate as it is possible to obtain them in experiments with small animals. In Series I the consumption was equalised within 2 g. per rat in

Table I. *Food consumption.*

Per rat in five weeks.

Cereal additions	Average total intake (g.)			Range of total intakes (g.)		
	I*	II†	III*	I*	II†	III*
Yellow maize	242	281	218	All 242	242-305	All 218
Rolled oats	242	234	217	240-244	190-265	200-221
Whole wheat	241	285	216	241-242	224-301	205-218
Patent flour	240	250	218	237-242	215-289	216-218
Polished rice	—	235	213	—	203-262	201-220

* Equalised consumption.

† *Ad libitum*, recorded consumption.

each group for the entire period of five weeks, with a maximum range of 7 g. between all the rats of the series. In Series III the consumption was equalised within 5 g. per rat in the five-week period, with a maximum range of 20 g. between all the rats in the series, with the exception of one. This rat, No. 816, ate only 160 g. instead of the approximate average of 200 g.; it therefore, being obviously abnormal, was omitted from the calculations. In Series II, which was run with *ad libitum* consumption, the maximum variation was much larger. It amounted to 51 g. as the average between the groups, with a maximum range between all individuals in the series of 201 g.

The different rations were not always consumed with the same facility in each series. In Series I, the consumption of rolled oats was the most limiting factor; patent flour next and yellow maize the least. In Series II polished rice and rolled oats were consumed the worst, with yellow maize and whole wheat the best. In Series III polished rice was again the most limiting, with rolled oats next in order and yellow maize the least.

From certain points of view, the value of attempting to equalise consumption may be questioned. It is a well-known fact after the observations of McCollum and co-workers that the severity of rickets produced on a rachitic diet is increased by the amount of ration consumed, but at the same time it is not clear whether this increased severity of rickets is due to the fact that the greater consumption of food causes greater growth and therefore greater impoverishment of bone in calcium and phosphorus; or whether the greater growth is the responsible factor and greater consumption of ration merely follows as an effect. E. Mellanby and Green have repeatedly pointed out how increased consumption of cereal increases the severity of rickets [1921: 1925; 1928], but in their latest paper they apparently placed more emphasis upon maintaining uniformity of growth than upon maintaining uniformity of consumption of ration.

In analysing these results, Green and E. Mellanby [1928] state that it was very difficult to obtain uniformity of growth because after the first few weeks the animals varied decidedly in their appetites. As examples of variation in weight, in Table I, the maximum weights attained varied from 47 to 94 g.: in Table II, for Series I they ranged from 66 to 106 g., and for Series II, 68 to 96 g.: in Table III from 51 to 81 g.: in Table IV from 50 to 113 g.: in Table V from 47 to 100 g. *etc.* From their data we are therefore forced to conclude that Green and E. Mellanby [1928] accomplished neither equalised consumption nor equalised growth. We, ourselves, are of the opinion that whatever may be the fault of limiting our experiments to equalised consumption, inasmuch as we were successful in accomplishing this purpose, our data put us in the position to evaluate this technique and later to extend our endeavours to secure rigid control over growth as well. Though this is admittedly difficult, if it is successfully accomplished, we shall then be able to compare the two factors.

Growth.

Our present data on growth obtained by taking increases in body weight are presented in Table II. We are not entirely satisfied with this technique because

Table II. *Summary of average growth values.*

Cereal additions	Rat weights											
	Series I				Series II				Series III			
	Weights (g.)			Maximum increase %	Weights (g.)			Maximum increase %	Weights (g.)			Maximum increase %
	Initial	Maximum	Final		Initial	Maximum	Final		Initial	Maximum	Final	
Yellow maize	59.2	95.0	95.0	60.3	57.8	99.7	99.7	72.5	57.2	70.2	75.5	38.5
Rollod oats	60.0	104.0	104.0	73.3	58.5	88.5	87.7	51.3	58.5	78.5	75.8	34.2
Whole wheat	59.0	91.8	91.8	55.6	58.2	98.5	98.5	69.2	58.6	79.0	74.7	34.4
Patent flour	57.2	92.5	92.5	61.7	58.2	87.3	87.0	50.0	58.0	81.7	76.3	40.9
Polished rice	Not included in Series I				58.3	91.0	91.0	56.1	59.3	84.0	83.8	41.7

it assumes that the increase in weight represents symmetrical body growth which is not necessarily the case, but we accept it for the time being. It has already been mentioned that all rats in the series, inasmuch as there were litter representatives in each group, have the same average ages for the group. The initial average weights, for all practical purposes, were also the same. Without going into detail, the differences in average initial weight between groups were 2.8 g. for Series I, 0.7 g. for Series II and 2.1 g. for Series III. This we believe represents an accurate measure of the uniformity of our rats as used for the different cereal experiments. This also stands in marked contrast to the animals used by Green and E. Mellanby [1928] who, as their tables show, used animals weighing from 29 to 52 g. in their various experiments in which they compared one cereal with another. They also made no statements in regard to initial ages of their animals.

Although, as stated before, we made no direct attempt to obtain uniformity of growth, this was practically accomplished unintentionally as seen by inspection of the table for maximum weights attained and for percentages of resultant increases in weight. In Series I the average maximum weights range from 91.8 g. for whole wheat as the minimum to 104 g. for rolled oats as the maximum. This gives percentages of increase of 55.6 and 73.3 respectively. In Series III the range of average maximum weights was from 78.5 g. for rolled oats as the minimum to 84.0 g. for polished rice as the maximum. Calculated as percentages of increase this gives values of 34.2 to 41.7 respectively. The resultant growth for Series II (*ad libitum* consumption) showed no marked difference from those obtained in Series I and III: the average maximum weights ranged from 87.3 g. for patent flour to 99.7 g. for yellow maize, giving a range of percentage increase of 50.0 to 72.5. It is to be added that such comparable results were unexpected because originally we had anticipated that Green and E. Mellanby's results might be due to differences in consumption of ration secured with their *ad libitum* technique.

The range of difference of percentage increase in weight was greater in the experiments of Green and E. Mellanby than in our own. For instance, in Table II under Series I their increases range from 100% in the case of oatmeal to 186% with white flour. In Series II they obtained increases in weight from 112% in the case of rice to 217% with white flour, and in Table III from 10% with rice to 145% with white flour. They point out that in the majority of cases in which rice was used, growth was so poor that it did not allow a good comparison with other cereals.

Green and E. Mellanby emphasised that experiments with rickets-producing cereals should be ended, if possible, during the period of growth and not prolonged when the animals have begun to lose weight. We agree with them that this is a desideratum. We practically accomplished what they considered essential, except for Series III. There we did encounter some losses in weight. These losses, when averaged for the different rats, amounted to only 4.2 g. We have no way of knowing whether or not Green and E. Mellanby came closer to meeting the above desirable conditions, because they presented only maximum weights in their tables. The difficulty that they experienced in securing consumption with rice, resulting in a maximum weight increase in one case of only 10% above the initial weight of the animals in 30 days, leads us to suspect that they had larger losses because in our experiment we obtained far better performance. We are unable to account for the losses in weight experienced by our rats in Series III, but whatever their origin our results are not influenced by it *in toto* because in Series I and II increases in weight were observed to the end.

Epiphyses and costochondral junctions.

The comparative degree of rachitic involvement of the epiphyses of the distal ends of the radii and ulnac, and of the costo-chondral junctions are presented in Table III. Numbers from 1 to 5 are used to indicate the progressive severity of

Table III. *Epiphyses and costo-chondral junctions (individual records)*.*

Cereal additions	Epiphyses*			Costochondral junctions†		
	I	II	III	I	II	III
Yellow maize	1 1	4 1	1 1	0 0	0 0	0 0
	2 1	3 3	1 1	3 0	0 0	0 0
		3 3	1 2		0 0	0 3
Rolled oats	1 1	1 1	0 1	0 0	0 0	0 0
	1 1	1 1	1 1	0 0	0 0	3 0
		0 1	1 1		0 0	0 0
Whole wheat	1 1	1 0	0 1	0 0	0 0	3 3
	1 1	0 1	1 1	0 0	3 3	0 0
		1 2	1 1		0 0	0 0
Patent flour	2 1	1 2	1 1	3 0	0 0	0 0
	2 1	1 0	1 1	3 0	0 0	0 3
		1 1	1 1		0 0	3 0
Polished rice	—	1 1	1 2	—	0 0	3 0
		1 1	1 2		0 0	3 0
		1 1	1 1		0 0	3 0

* Condition of epiphyses: 0 denotes normal, 1 denotes narrow rachitic, 2 denotes medium rachitic, 3 denotes medium to wide rachitic, 4 denotes wide rachitic, 5 denotes very wide rachitic.

† Conditions of costo-chondral junctions: 0 denotes normal, 3 denotes moderate beading, 5 denotes very marked beading.

the lesions with 0 for the normal. In general the rickets produced was not of a severe type because it did not exceed stage 3, which represents only a moderate rickets. The yellow maize group in Series II stands out from the others in having one animal in stage 4 and more than any others in stage 3, but the costo-chondral junctions in this group were all normal. It may be assumed that variations in beading can result from differences in behaviour of individuals with resultant variations in strain and hyperplasia of the rib junctions. Possibly it may also be granted that the widths of the metaphyses are determined by changes in the rapidity of growth from time to time as the deposition of mineral elements and concomitant growth of ossein fails to keep pace. We have noted, for instance, that with cessation of growth in severe rickets the metaphyses do not increase in width even though the rachitic condition as judged by other criteria becomes more severe. It is noteworthy that the average growth on the yellow maize in the aforementioned instance was greater during the last week than in other serial groups.

Femora.

In a comparative macroscopic study of the severity of rickets, probably the most significant data to consider are those concerned with the size of the bones and their mineralisation. In this respect we have employed the alcohol-extracted dried weight, the total ash weight, and the percentage of ash in the femora. The values of percentage of ash contained in the femora were calculated on the basis of their alcohol-extracted dried weights. Green and E. Mellanby [1928] used as indices the percentage of calcium in the fat-extracted dried bones, and the A/R ratio, where A represents the weight of bone ash and R the difference between the weights of the fat-extracted dried bone and the ash. In our experience with the data of these experiments and those of Green and E. Mellanby, a very high positive correlation was found to exist between the percentage of calcium, percentage of ash, and the A/R ratio. Consequently, each may serve equally well as a measure of the degree of rachitic involvement.

In Table IV we have reported the data for Series I. Attention is called particularly to weight of the total ash and percentage of ash in the femora. These data are typical of the ranges of variation shown in all three series. We have

Table IV. *Detailed data of Series I on femur analyses.*

Rat no.	Initial weight and sex g.	Total gain g.	Average consumption daily g.	Femora		
				Dry weight mg.	Ash weight mg.	% ash
Yellow maize						
544 <i>a</i> *	62 ♂	36	6.9	117.1	51.4	43.9
545 <i>b</i>	63 ♀	32	6.9	115.1	52.3	45.5
546 <i>c</i>	55 ♂	43	6.9	121.7	55.7	45.8
547 <i>d</i>	57 ♀	32	6.9	111.4	50.8	45.6
Rolled oats						
548 <i>a</i>	61 ♂	52	7.0	135.4	58.7	43.4
549 <i>b</i>	60 ♀	31	6.9	127.6	58.15	45.5
550 <i>c</i>	61 ♂	45	6.9	148.9	70.8	47.8
551 <i>d</i>	58 ♀	48	6.9	126.7	59.1	46.6
Whole wheat						
552 <i>a</i>	62 ♂	32	6.9	126.5	56.7	44.8
553 <i>b</i>	60 ♀	29	6.9	131.5	63.7	48.4
554 <i>c</i>	57 ♀	35	6.9	125.1	60.1	48.0
555 <i>d</i>	57 ♀	35	6.9	132.4	62.2	47.0
Patent flour						
556 <i>a</i>	58 ♂	41	6.9	133.6	53.3	39.9
557 <i>b</i>	56 ♀	36	6.9	126.7	52.5	41.4
558 <i>c</i>	58 ♀	34	6.8	112.1	48.4	43.2
559 <i>d</i>	57 ♀	30	6.9	121.2	52.8	43.6

* Letter exponents refer to litter-mates.

summarised in Table V only the average values for each cereal. Considering the data for extracted femur weights of the three series *in toto*, it is difficult to rank the various cereals in any definite order except to recognise that yellow maize produced the lightest bone.

If we average the weights of ash for our three series of experiments we obtain for maize, oats, wheat, flour and rice respectively 47, 50, 52, 49 and 48 mg., and if we average only the values for Series I and III in which the food consumption was equalised we obtain quite similar values, *viz.* 47, 51, 52, 50 and 48 mg.

Table V. *Average femur analyses.*

Cereal additions	Rat femora											
	Average dry weight* (mg.)			Average ash weight (mg.)			Average ash %			A/R ratio†		
	I	II	III	I	II	III	I	II	III	I	II	III
Yellow maize	116.3	114.7	106.6	52.6	46.5	43.6	45.2	40.5	40.9	0.83	0.68	0.69
Rolled oats	134.1	117.6	108.5	61.7	47.4	45.3	45.9	40.4	41.7	0.85	0.58	0.72
Whole wheat	128.9	127.4	109.7	60.7	51.2	47.1	47.1	40.3	42.8	0.80	0.67	0.75
Patent flour	123.4	120.9	115.2	51.8	48.9	49.0	42.0	40.5	42.5	0.72	0.67	0.74
Polished rice	—	122.9	114.2	—	51.6	48.4	—	42.0	42.3	—	0.72	0.74

* After fat extraction.

† A, denotes ash weight of a bone "X"; R, the difference in weight between the fat extracted dried bone "X" and its ash.

Calculating the average percentage of ash in a similar manner, we obtain 41, 42, 42, 41 and 42 % respectively in the first case and 42, 43, 44, 42 and 42 % in the second. These differences certainly do not appear impressive.

Blood analyses.

Since Gutman and Franz [1921-22] and Kramer and Howland [1922] showed that either low calcium or low inorganic phosphorus values of blood serum of rats could be used as an index of the severity of rachitic lesions, many investigators have determined these values. Whilst the importance of these as an index is now discounted to some extent [Bethke *et al.*, 1923], and justifiably so, they were nevertheless obtained in our analyses.

Kramer and Howland [1922], McCollum *et al.* [1922, 1], Cavins [1924], Dutcher *et al.* [1925] originally demonstrated with rachitic rats showing sub-normal calcium or inorganic phosphate values for blood serum, that starvation results in an increase of these elements in the blood stream. If fasting had occurred it is possible that marked discrepancies would have been introduced into our data. We however need give no consideration to this factor since none of the rats on any of the cereals ever refused food and they were eating daily 9, 6 to 8, and 5 g. respectively at the end of the five weeks of experiment.

The normal calcium values for rat blood serum have been reported by Kramer and Howland [1922], Bethke *et al.* [1923] and by Cavins [1924], as ranging from 9.5 to 10.5, 10.5 to 13.6 and 9.5 to 10.0 mg., respectively, per 100 ml. of serum. The calcium values of the blood sera of all the rats in Series I, II and III, presented in Table VI, are conspicuously below the normal values and range

Table VI. *Calcium and inorganic phosphorus of sera.*

Cereal additions	Ca mg. per 100 ml. serum*			P mg. per 100 ml. serum*			Ca x P per 100 ml. serum*		
	I	II	III	I	II	III	I	II	III
Yellow maize	6.80	4.40	4.28	7.45	8.88	7.25	50.7	39.1	31.0
Rolled oats	5.85	3.80	4.63	8.15	9.04	8.21	47.7	34.4	38.0
Whole wheat	6.60	4.40	4.02	8.10	10.37	8.15	53.5	45.6	32.8
Patent flour	Lost	4.40	3.98	Lost	9.77	9.10	Lost	43.0	36.2
Polished rice	—	4.25	3.94	—	8.65	7.69	—	36.8	30.3

* Sera from pooled bloods.

from a minimum of 3.8 mg., to a maximum of 6.8 mg. per 100 ml. of serum. The rolled oats diets produced the lowest values in Series I and II and the highest in Series III. The calcium values of the remaining cereals compared in each series differ so little that they may be considered identical. In fact, the striking similarity of all these comparative calcium values again points to the approximate equality of these cereals in the production of a mild form of rickets.

The normal inorganic phosphate values for the serum of rat blood have been reported by the same aforementioned authors and by Dutcher *et al.* [1925] as ranging from 7.0 to 8.5, 9.0 to 9.5, 7.0 to 8.5 and 8.0 to 10.0 mg., respectively, per 100 ml. of serum. The inorganic phosphate values of the sera of Series I, II and III, Table VI, range from 7.25 to 10.37 mg., all of which lie within or slightly above the reported normal ranges. Those for yellow maize and polished rice are the lowest while those for whole wheat and patent flour are the highest.

The separate calcium and inorganic phosphate values of the blood sera also lend themselves to the calculation of additional values which may be used further to compare the cereals. Kramer and Howland [1923] demonstrated that the products of these calcium and inorganic phosphate values in mg. per 100 ml. could be used to indicate the severity of rickets. They concluded that when the product is below 30 rickets is to be expected, when between 30 and 40 it is probable. When above 40 rickets is either healing or entirely absent. The calcium and phosphorus products of Series I, II and III range from 47.7 to 53.5; 34.4 to 45.6; and 30.3 to 38.0, respectively. In Series I and II the largest products are shown by whole wheat and the smallest by rolled oats, but in Series III rolled oats has the largest and both yellow maize and polished rice the smallest values. Since figures obtained as the products of two or more values exaggerate uncontrollable small variations, we believe no significant differences are revealed by these calcium and phosphorus products.

Intake of calcium and phosphorus.

Cereals in general are known to be very low in both calcium and the antirachitic factor and to contain only moderate amounts of phosphorus. Dibbelt [1909] produced rickets in dogs by feeding diets deficient in calcium. Sherman and Pappenheimer [1921] and McCollum, Simmonds and Kinney [1922] produced rachitic bones in rats on diets low in calcium and the antirachitic factor. McCollum *et al.* [1921, 1, 2; 1922, 2] and Sherman and Pappenheimer emphasised the importance, for normal bone formation, of maintaining a definite relationship between the calcium and phosphorus in the diet. This belief is probably accepted more absolutely than is justifiable because much depends on the amounts of each in the diet [Brown *et al.*, 1932] and as we know now on the form in which they are present [Bruce and Callow, 1934].

Calcium and phosphorus determinations were made on all our rations. The calcium was determined by a volumetric adaptation of the McCrudden method [1911-12] and the phosphorus gravimetrically by precipitation as ammonium phosphomolybdate and magnesium ammonium phosphate. The calcium and phosphorus analyses per 100 g. of ration are shown in Table VII.

The intake of phosphorus per 100 g. of ration is not markedly different from the requirements of 494 mg., originally reported by Shipley [1922] as being normal. It ranged between 70 and 118% of this value. The rations of yellow maize supplied the requirements; those of rolled oats and whole wheat exceeded these by 18%; and those of patent flour and polished rice were too low by 30%.

The normal amount of dietary calcium required for continuous growth, maintenance and normal function in rats, when all other dietary factors are

Table VII. *Calcium and phosphorus intake and calcium-phosphorus ratios in the rations.*

Cereal additions	Total intake (per rat)						Intake per 100 g. ration					
	Ca (mg.)			P (mg.)			Ca (mg.)		P (mg.)			
	I	II	III	I	II	III	I	II and III	I	II and III	Ca/P	
											I	II and III
Yellow maize	195	185	143	1196	1268	985	81	66	494	452	1:6.1	1:6.9
Rolled oats	273	231	214	1398	1347	1246	113	99	579	574	1:5.1	1:5.8
Whole wheat	274	283	214	1399	1642	1242	111	99	580	576	1:5.1	1:5.8
Patent flour	210	183	159	839	862	749	87	73	349	344	1:4.0	1:4.7
Polished rice	—	150	130	—	808	699	—	64	—	343	—	1:5.4

supplied, has been reported by Shipley [1922] to be 640 mg. per 100 g. of ration. Casual inspection immediately shows all rations to be low in calcium. They supplied only between 10 and 18 % of the normal amount required, according to this standard. The intakes of this element per 100 g. of ration ranged in Series I from 81 to 114 mg. and in Series II and III from 64 to 99 mg. In the first series approximately 38 % less calcium was furnished by the yellow maize and patent flour rations than by those of rolled oats and whole wheat. In the second and third series alike about 47 % less calcium was supplied by the yellow maize, patent flour and polished rice rations than by those of rolled oats and whole wheat. In all the series the yellow maize rations have the widest ratios, those of patent flour the narrowest and those of polished rice, rolled oats and whole wheat practically identical intermediate values.

SUMMARY.

Rolled oats, patent flour, whole wheat, polished rice and yellow maize when making up 75 % of a low-calcium ration have been compared in their capacity to produce rickets in the rat. The severity of the rachitic lesions produced by these different cereals and cereal products were remarkably similar. In this respect our findings are at variance with those of Green and E. Mellanby [1928] who contend that cereals differ markedly in their capacity to produce rickets, and that oatmeal is the worst offender and white flour the least.

Our conclusions are based on the extent of mineralisation in the distal ends of the radii and ulnae; the degree of hyperplasia of the costochondral junctions; the total weights, ash weights and percentage ash of the fat-extracted femora; and the calcium and inorganic phosphate relations of the blood sera in contrast with the percentage of calcium and the *A/R* ratio of the leg bones used by Green and Mellanby.

In a previous paper [Steenbock *et al.* 1930] we have presented results which showed that maize, wheat and oats fed with other supplements than those used in these experiments differed somewhat in their bone forming capacities: wheat being the best, oats the next best, and maize the poorest. This brings out the modifying effect of supplements without the use of which the calcifying—or anticalcifying—values of cereals cannot be tested.

It is obvious that generalisations as to the specific bone-building value of different cereals from experimental results with rats are beset with many uncertainties.

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XXIX. THE EFFECT OF SELENIUM ON CELLULAR METABOLISM. THE RATE OF OXYGEN UPTAKE BY LIVING YEAST IN THE PRESENCE OF SODIUM SELENITE.¹

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RECENT reports indicate that selenium is chiefly responsible for numerous cases of livestock poisoning in certain parts of the United States [Byers, 1935]. Franke and Potter [1935] have shown that rats develop serious symptoms and almost invariably die on a diet containing as little as 30 parts per million of selenium in the form of sodium selenite. The majority of the animals became extremely anaemic before death.

Moxon and Franke [1935] found that traces of selenium will greatly reduce the fermentation of glucose by yeast although Harden and Norris [1914] found that 1 % Na_2SeO_3 slightly increased the rate of fermentation of glucose by top brewery yeast. Yeast cells are apparently as sensitive to the presence or absence of traces of certain elements as are the higher organisms. In the latter deficiency of iron and copper will result in anaemia due to diminished haemoglobin formation [Hart *et al.*, 1928; Elvehjem and Hart, 1929] whilst, in the case of yeast, Elvehjem [1931] has shown that the rate of oxygen uptake is decreased appreciably by such deficiency.

In the present investigation the authors decided to study the oxygen uptake of living yeast cells in order to determine whether or not the symptoms of selenium poisoning might be fundamentally due to an inhibition of the processes of tissue respiration. It was also felt that selenium might prove to be a valuable tool in the study of the respiratory mechanisms of normal cells.

The respiratory mechanisms in yeast are evidently somewhat simpler than those in most animal tissues since the fact that the oxygen uptake in yeast is practically completely inhibited by cyanide [Dixon and Elliott, 1929] indicates that all the dehydrogenase systems are anaerobic. In the present investigation frequent control cyanide-inhibition experiments were made. Preliminary experiments have shown that selenium does not cause as complete inhibition of the oxygen uptake of yeast as cyanide. This suggests that selenium does not inactivate the cytochrome or indophenol oxidase but rather that it attacks certain dehydrogenating systems.

After a number of experiments to determine the effect of variations in the suspending medium, the authors studied the oxygen uptakes of normal and poisoned yeast cells on a variety of substrates, in order to demonstrate any specific toxicity that might exist for particular enzyme-substrate systems.

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² Wisconsin Alumni Research Foundation Fellow.

Preliminary experiments.

The oxygen uptake was measured by means of a Barcroft differential respirometer as previously described by Starc and Elvehjem [1933]. To each flask were added 1.0 ml. of buffer solution and 1.0 ml. of substrate solution. 1 ml. of water was added to the left-hand flask and 1 ml. of yeast suspension to the right-hand flask. The yeast¹ was suspended in redistilled water and shaken until a homogeneous suspension resulted. The quantity of yeast used was determined by preliminary experiments and was that amount which would produce the greatest oxygen uptake within the range of the apparatus when glucose was used as substrate. The proper concentration (5 mg. yeast per ml.) was accordingly used for all the experiments reported in this paper. When poisons were being tested, 0.1-0.3 ml. of poison was added to both flasks, and the amount of buffer was decreased so that the final volume was always 3 ml.

The buffer used was a mixture of equal parts of 0.15 *M* NaH_2PO_4 and KH_2PO_4 which had been brought to p_{H} 6.5 by addition of NaOH and KOH respectively. Since 1 ml. of buffer was used, the concentration in the respirometer flasks was 0.05 *M*. Elvehjem [1931] and Dickens [1934] have measured the oxygen uptake of yeast in 0.05 *M* sodium phosphate, which was adjusted to p_{H} 7.0 in the former case. In the present study no appreciable change in the uptake was noted when the buffer concentration was diminished to 0.025 *M*. The first experiments were carried out in sodium phosphate; when potassium phosphate was substituted it was observed that the rate of uptake increased with time, whereas the rate had either decreased or remained fairly constant when sodium was the cation. Since K^+ seemed to have a stimulating effect upon the uptake, the buffer which was finally used was a mixture of sodium and potassium phosphates. At this time Ashford and Dixon [1935] published a statement that K^+ increased aerobic glycolysis in brain slices but had no effect upon the oxidative removal of lactic acid. Lasnitzki and Szörenyi [1935] found that K^+ increased yeast fermentation, and that Rb^+ and Cs^+ also had more marked effects than Na^+ or Li^+ . Dickens and Greville [1935] have since studied the effects of various cations on tissue respiration in some detail and report that K^+ , Rb^+ and Cs^+ produce a greater general stimulation of carbohydrate respiration in brain tissue than Li^+ or Na^+ . The effect however could not be demonstrated in other tissue. Our work shows that yeast respiration is also susceptible to the so-called "potassium effect", the respiration on glucose being greatly accelerated.

Ehrenfest [1932] and Schroeder *et al.* [1933, 1] showed that iodoacetate inhibition of yeast fermentation was dependent upon the p_{H} of the suspending medium. They found that the toxicity decreased as the medium became less acid, and attributed their results to a change in the permeability of the yeast cells. Our results show a similar relation between p_{H} and degree of inhibition.

Seven phosphate buffers were made up of p_{H} 4.5 to 7.5. The results of a typical experiment are shown in Fig. 1. The uptake in the control flasks was greatest at p_{H} 6.0 and 6.5 and was somewhat less at 4.5 and 7.5. The Q_{O_2} ($\mu\text{l. O}_2$ uptake per g. of dry yeast per hour) of the yeast on the glucose substrate has been calculated to be approximately 100,000, on the basis of the experiments lasting 2 hours. This is over twice as great as has been previously reported [Elvehjem, 1931; Dixon and Elliott, 1929] and may be explained partly by the freshness of the yeast and partly by the fact that the buffer may be nearer the optimum. It

¹ Fresh pressed yeast was obtained from the Department of Agricultural Bacteriology through the courtesy of Dr I. L. Baldwin. Analysis showed the moisture content to be quite uniform at 71-73 %.

was found that the p_H of the buffer had a profound effect on the degree of inhibition produced by $M/300$ selenite. From p_H 4.5 to 6.5 the inhibition was maximum, but as soon as neutrality was passed it decreased rapidly. At p_H 7.5 inhibition was very slight, whilst at p_H 7.0 the uptake was between values obtained at p_H 6.5 and 7.5. A large number of experiments uniformly confirmed these results. However, when the selenite concentration was increased to $M/100$, the p_H effect was not observed, probably owing to the large excess of poison. A few experiments were made with sodium arsenite but no diminution in toxicity was observed at p_H 7.5 using arsenite at concentrations of $M/300$, $M/1000$ or $M/3000$.

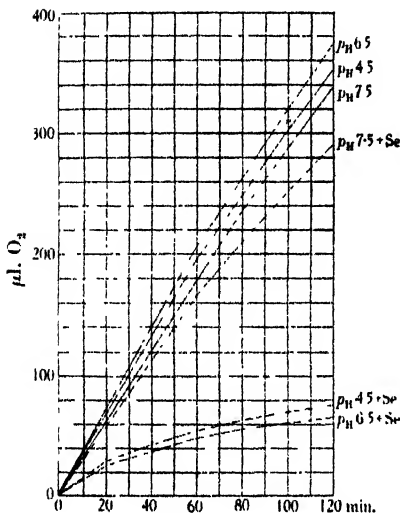


Fig. 1.

Fig. 1. The effect of p_H on selenium toxicity, Series 38, 5 mg. yeast, 0.05 M NaH_2PO_4 , $M/18$ glucose, $M/300$ Na_2SeO_3 .

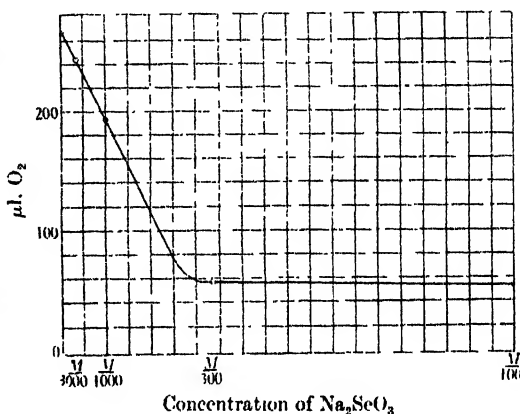


Fig. 2.

Fig. 2. Oxygen uptake at various concentrations of selenium, $M/18$ glucose, p_H 6.5, 0.05 M sodium and potassium phosphate buffer, 5 mg. yeast, 2-hour period, Series 52, 69 A1, 69 A2, 70 A, 70 B.

A number of experiments were carried out to determine the proper concentration of sodium selenite. The results are summarised in Fig. 2. It was found that the minimum effective concentration was $M/300$, whilst $M/100$ produced very slight additional inhibition. The selenite was prepared at a concentration of 0.1 M and 0.1 ml. was added to the flasks to make the final concentration in the solution $M/300$. The sodium selenite used¹ had been previously shown to be of high purity [Franke and Potter, 1935] and in addition was shown to have the same order of toxicity as a laboratory preparation.² The arsenic used was in the form of sodium arsenite and was tested at the same level as the selenium, although later experiments have shown that its minimum effective concentration is lower than that of the selenite, being approximately $M/1000$.

The experiments were carried out at two temperatures, 28 and 37°. Although

¹ Emmer and Amend, C. P. Anhydrous.

² Furnished through the kindness of Dr V. W. Meloche, Dept. of Chemistry, University of Wisconsin.

the uptake is more rapid at 37°, the lower temperature is probably nearer the optimum for yeast [Moxon and Franke, 1935] and most of the experiments were carried out at that temperature.

Substrate experiments.

After the preliminary work on experimental conditions, a number of experiments were carried out using various substrates. The oxygen uptake of yeast is largely determined by the substrate. The data in Tables I and III show that the

Table I. *Inhibition of oxygen uptake on glucose substrate.*

Temperature 28°. Buffer: $\text{NaH}_2\text{PO}_4 + \text{KH}_2\text{PO}_4$ neutralised to p_{H} 6.5.

Poison	% inhibition at 2 hours	$\mu\text{l. uptake per 5 mg. yeast}$	
		0-60 min.	60-120 min.
<i>M</i> /300 Se	73	48.2	23.4
Control	—	119.5	141.0
<i>M</i> /300 As	83	26.4	18.1
Control	—	117.5	144.5
<i>M</i> /300 Se	57*	61.0	36.2
Control	—*	120.5	106.5
<i>M</i> /300 Se	56*	48.6	30.4
Control	—*	87.0	94.0
<i>M</i> /300 Se	79	44.2	20.8
Control	—	135.0	176.0
<i>M</i> /300 Se	85†	43.5	20.3
Control	—†	207.0	224.0
Control	—	133.5	163.5
<i>M</i> /25 NaF	2	134.0	156.0
<i>M</i> /75 NaF	—	140.0	177.0
Control	—†	128.0	108.0
<i>M</i> /25 NaF	10†	124.0	143.0
<i>M</i> /75 NaF	1†	128.5	165.5
Control	—	119.8	148.5
<i>M</i> /100 Se	82	31.2	16.3
<i>M</i> /300 As	85	26.4	17.6
Control	—	131.7	164.0

* Red Star Commercial Yeast Cake.

† 37°.

‡ p_{H} 7.5.

Table II. *Inhibition of oxygen uptake on lactate substrate.*

Temperature 28°. Buffer: $\text{NaH}_2\text{PO}_4 + \text{KH}_2\text{PO}_4$ neutralised to p_{H} 6.5.

Poison	% inhibition at 2 hours	$\mu\text{l. uptake per 5 mg. yeast}$	
		0-60 min.	60-120 min.
<i>M</i> /300 Se	7	89.3	74.2
Control	—	94.2	79.3
<i>M</i> /300 As	—*	9.3	5.4
<i>M</i> /300 Se	8*	47.6	22.6
Control	—*	48.9	27.5
<i>M</i> /300 Se	7*	39.4	25.1
Control	—*	39.4	29.6
<i>M</i> /300 Se	5	92.0	79.0
Control	—	92.8	86.2
<i>M</i> /300 Se	7†	140.3	110.7
Control	—†	149.7	119.0
Control	—	87.4	110.6
<i>M</i> /300 Se	12	82.3	91.9
<i>M</i> /300 Se	—	89.4	94.3
<i>M</i> /300 As	—	11.4	3.4
<i>M</i> /300 As	93	9.3	4.6
Control	—	87.1	99.9

* Red Star Commercial Yeast Cake.

† Temperature 37°.

Table III. *Inhibition of oxygen uptake on various substrates.*Temperature 28°. Buffer: $\text{NaH}_2\text{PO}_4 + \text{KH}_2\text{PO}_4$ neutralised to p_{H} 6.5.

Substrate	Poison	%	$\mu\text{l. uptake per 5 mg. yeast}$	
			0-60 min.	60-120 min.
No substrate	Control	—	17.2	19.4
"	$M/300 \text{ Se}$	77	6.1	2.2
"	$M/300 \text{ As}$	87	2.8	1.7
"	Control	—	19.1	14.7
Succinate ($M/9$)	$M/300 \text{ Se}$	73	5.9	3.1
"	Control	—	12.7	20.0
Acetate ($M/9$)	Control	—	33.7	87.8
"	$M/300 \text{ Se}$	56	34.1	19.3
"	Control	—	25.7	58.3
"	$M/300 \text{ Se}$	39	31.1	20.5
"	Control	—	30.3	80.4
"	$M/300 \text{ Se}$	44	36.1	25.7
"	Control	—	23.5	57.1
"	$M/300 \text{ Se}$	46	26.1	17.6
"	$M/300 \text{ As}$	97	1.7	1.9
"	Control	—	45.8	74.9
Ethyl alcohol ($M/9$)	Control	—	62.8	96.9
"	$M/300 \text{ Se}$	70	34.3	13.7
"	$M/300 \text{ As}$	72	42.5	27.9
"	Control	—	109.7	140.3
Fructose ($M/18$)	Control	—	168.8	234.2
"	$M/300 \text{ Se}$	84	43.6	21.7
"	$M/300 \text{ As}$	89	15.6	12.3
"	Control	—	112.8	148.9
Mannose ($M/18$)	Control	—	91.6	130.4
"	$M/300 \text{ Se}$	76	32.1	21.1
"	$M/300 \text{ As}$	86	26.4	15.3
"	Control	—	130.5	177.8
Pyruvate ($M/9$)	Control	—	65.7	90.3
"	$M/300 \text{ Se}$	4	64.6	85.2
"	$M/300 \text{ As}$	97	4.1	2.3
"	Control	—	85.7	98.8
"	$M/300 \text{ Se}$	9	51.9	70.9
"	Control	—	55.8	78.8

uptake by the yeast alone is only 10-15 % of the uptake when glucose is present. It is thus possible to determine the ability of the yeast organism to oxidise specific compounds.

Elvehjem [1931] and Dickens [1934] have used $M/18$ glucose as a substrate and this concentration was found to be quite satisfactory in the present investigation. The uptake was not affected by reducing the concentration of the sugar to $M/36$. The concentrations of the other substrates were based on the glucose solution. The substrate solutions were prepared fresh each day, with the concentration three times as great as the final concentration in the respirometer flasks. In these experiments the amount of yeast was so small (5 mg.) that the substrate concentration was not greatly altered during the period of the experiment. The buffer was also strong enough to hold the p_{H} constant.

The substrates were all C.P. chemicals, and in the case of the alcohol and pyruvic acid were repurified. The mannose and fructose were laboratory preparations.¹

¹ The mannose and fructose were kindly supplied from the laboratory of Dr Karl Paul Link.

Most of the substrate experiments were done in triplicate, and the figures reported are the average values. In general, individual manometers agreed within 5%. The uptake was studied for a period of 2 hours, and readings were taken at 20-min. intervals. The results are recorded in Tables I, II and III. Fig. 1 shows the general form of the curves for these data. A few experiments were continued for 6 hours, but the curves were merely extensions of those obtained at 2 hours and yielded no additional information.

It was observed that when glucose, fructose or mannose was used as substrate, the rate of oxygen uptake was reduced about 80% by the addition of selenite (Tables I and III). The results with a sample of commercial yeast showed less inhibition and are included in Table I to show that yeast from various sources may vary in the degree to which it is inhibited by selenium, just as in the case of cyanide [Dixon and Elliott, 1929]. As soon as lactate and pyruvate were used as substrates however it was found that the inhibition of oxygen uptake by selenite became negligible (4–9%). Moxon and Franke [1935] found that the rate of CO_2 evolution from glucose-yeast mixtures was greatly diminished by sodium selenite. Their results show that selenium prevents yeast from metabolising glucose in a normal manner. Our work seemed to indicate that selenium was acting primarily as an inhibitor of the glycolytic system, since it appeared that our results could be explained without postulating an inhibition of oxidative mechanisms, on the assumption that in order for glucose to be oxidised, it must be split into 3-carbon compounds. Barron and Miller [1932] have accumulated excellent evidence to show that this is actually the mechanism in the case of gonococci. It was shown that in the presence of sodium fluoride, which is regarded as a specific poison for the glycolytic system [Dickens and Simer, 1929; Meyerhof and Kieselring, 1933], these organisms were able to oxidise lactate and pyruvate but not glucose. Yeast cells however seem to possess a somewhat greater versatility than gonococci. When sodium fluoride was added to mixtures of yeast and glucose the oxidation proceeded normally (Table I) although the concentration of fluoride exceeded the amount which prevented oxygen uptake by gonococci. Since glucose dehydrogenase is not poisoned by fluoride [Harrison, 1931] there is thus some evidence that this enzyme is present in yeast. The point must remain obscure until the effect of fluoride on living yeast has been more thoroughly investigated. Thus far, most of the yeast glycolysis experiments have been carried out with dried yeast preparations, which may be expected to have lost many of the properties of living yeast. We cannot be certain that glycolysis was inhibited soon enough in our experiments completely to eliminate the formation of oxidisable intermediates. If yeast does in fact possess a glucose dehydrogenase, it is apparent that this part of the oxidative mechanism is poisoned by selenite.

The work with other substrates (Table III) furnishes definite evidence that selenium is toxic to the oxidative as well as the glycolytic systems. Since the uptake with yeast alone is relatively small even without selenium, and since the nature of the compounds being oxidised is unknown, little emphasis can be placed on inhibition in this case. Succinate is poorly oxidised and the uptake may be largely endogenous. However, there is definite evidence that yeast oxidises acetic acid and alcohol and that the oxidation is largely inhibited by selenite.

Arsenite is apparently much more toxic to the oxidative mechanisms than selenite, and produced marked inhibition with all substrates, including lactate and pyruvate. A few experiments were carried out to show that the percentage inhibition was of the same magnitude with lactate as with glucose even when the

arsenite was reduced to concentrations of $M/1000$ and $M/3000$. Thus, arsenic and selenium, although generally described as being pharmacologically similar, actually appear to show fundamental differences in their modes of action.

The inhibition of oxygen uptake by selenite appears to be a time reaction and is quite different in type from the inhibition which is produced by cyanide. The latter blocks the oxygen-carrying activity of cytochrome and exercises its maximum effect at once. Selenite, on the other hand, becomes increasingly toxic with time. It seems unlikely that this delayed action can be explained on the basis of permeability, since increasing the selenite concentration from $M/300$ to $M/100$ increases the inhibition but slightly.

It is a well-known fact that living cells, including yeast, can reduce selenites to selenium. It seems quite possible that the toxic properties of sodium selenite may be related to its ability to react with sulphhydryl groups. Bersin [1935] states that selenite reacts with glutathione, with the intermediate formation of G.S.Se.S.G which then decomposes into oxidised glutathione and free selenium. Voegtlin *et al.* [1931] have also observed the ability of selenium salts to catalyse the oxidation of glutathione. Schroeder *et al.* [1933, 2] have studied the relation between loss of sulphhydryl and inhibition of fermentation by iodoacetic acid. These workers observed that removal of glutathione from suspensions of poisoned yeast was a time reaction and showed that the loss was most marked in the acid range, being virtually negligible after the solution became neutral or alkaline. Their results may thus help to explain the relation between selenium toxicity and p_H . They also found that the decrease in sulphhydryl ran parallel with the degree of inhibition but thought that the decrease in glutathione could not be completely responsible for the results obtained. Geiger [1935] has recently done much toward clarifying the rôle of glutathione in glycolysis. He found that oxidised glutathione even in low concentrations inhibits lactic acid formation from both glucose and glycogen, and suggested that the glycolytic enzyme may be inactivated through oxidation by oxidised glutathione. It thus appears that selenite may inhibit glycolysis indirectly through the production of oxidised glutathione.

On the other hand, the failure of selenite effectively to inhibit the oxidations of pyruvate and lactate may be only apparent. The authors have not been able to prove conclusively that the selenite is not reduced by the substrates, although indications are that no reduction takes place. It is possible that lactate and pyruvate facilitate reduction of selenite by yeast cells and that glucose does not have this property.

At present the glutathione reaction seems to offer the best explanation for the toxic action of selenium, at least so far as glycolysis is concerned. Further work must be done with the intermediate and end-products of glycolysis to discover the real mechanism of the inhibition. Although it is apparent that the oxidative systems are also definitely inhibited, present data are too meagre to warrant any ideas as to mode of action.

SUMMARY.

1. The oxygen uptake of living yeast has been measured using the following substrates: glucose, mannose, fructose, lactic acid, pyruvic acid, acetic acid, alcohol and succinic acid in the absence and presence of sodium selenite.

2. Sodium selenite was found to produce about 80 % inhibition of the oxygen uptake on the sugars and less than 10 % inhibition on lactate and pyruvate substrates. $M/300$ was found to be the minimum effective concentration.

3. Inhibition by selenite was most marked on the acid side and fell off rapidly at p_H 7.0 and 7.5.

4. Parallel experiments with sodium arsenite indicated that this salt is considerably more toxic to the oxidative systems than selenite, the minimum effective concentration being about $M/1000$.

5. A possible mechanism for the inhibition of glycolysis by selenite is discussed.

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XXX. AMINO-ETHYL PHOSPHORIC ESTER FROM TUMOURS.

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THE biochemical importance of phosphoric esters in normal tissues is well recognised, the subject having been discussed fully in the *Annual Reviews of Biochemistry*. The phosphoric esters of malignant tumours have not been as thoroughly investigated and none of them has been positively identified.

The occurrence of an extremely soluble phosphoric ester in the filtrate from the basic lead acetate precipitation of a trichloroacetic acid extract of tumour was reported by Outhouse [1933]. This appears to be similar to the compound obtained by Booth [1935] from kidney, liver and brain. The author [1935] has recently reported the occurrence of this compound in twenty normal tissues as well as in a series of tumours, malignant and non-malignant. In addition, he reported two new phosphoric esters:

(1) a compound whose empirical formula suggests the phosphoric ester of an amino-hexahydric alcohol,

(2) a compound which appears to be an amino-ethyl phosphoric ester.

This paper is a report on the isolation, purification and identification of the latter compound.

Isolation and purification.

The material used was bovine malignant tumours, ranging in weight from 500 g. to 10 kg. They were minced and extracted with 3 volumes of ice-cold 4% trichloroacetic acid as soon as possible (generally about 2 hours) after the killing of the animal. The trichloroacetic acid extract was allowed to stand for 1 hour and then filtered. Powdered baryta was added to the filtrate until it was pink to phenolphthalein. The precipitate was centrifuged off and rejected. To the solution 5 g. of mercuric acetate per litre were added. The mercury precipitate was removed by centrifuging and basic lead acetate was added until no further precipitation of organic phosphate occurred.

The lead precipitate was removed by centrifuging, washed with water and decomposed with hydrogen sulphide. After centrifuging, the supernatant liquid was freed from H_2S by aeration, and baryta was added to it until p_H 10 was reached; it was then filtered into 4 volumes of 95% alcohol.

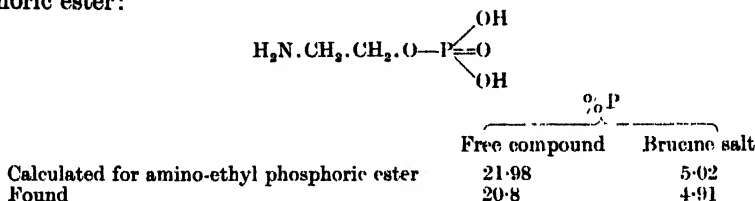
The precipitate was filtered off, washed with alcohol and with ether and dried. It was dissolved in 10 parts of water and twice reprecipitated from 4 volumes of alcohol. The barium salt was converted into the brucine salt and a brucine fractionation was made by crystallising the brucine salts from methyl alcohol to which increasing amounts of acetone were added.

The main fraction (No. 2), which accounts for 60% of the total phosphorus, was boiled in methyl alcohol. Dissociation of the brucine salt occurred and the precipitate obtained from the boiling showed 20.8% phosphorus. It was dissolved in water and reprecipitated from acetone. The phosphorus contents of the

Brucine fractionation.

Fraction	Wt. g.	P %
1	2.55	0.35
2	5.08	4.91
3	3.00	1.19
4	2.85	1.45
5	1.60	1.62
6	1.40	1.21

free compound and of the brucine salt indicated that the organic residue could contain two atoms only of carbon. This, taken in conjunction with the finding of a N : P ratio of 1 : 1 in the free compound, suggested it to be amino-ethyl phosphoric ester:

*Identification.*

To identify this compound it was considered advisable to prepare amino-ethyl phosphoric ester synthetically and to compare the naturally occurring compound with the synthetic. For further comparison flavianates of the two materials and of their hydrolytic products were prepared.

Preparation of amino-ethyl phosphoric ester. To 9 ml. amino-ethanol were added 3 ml. of orthophosphoric acid. A white crystalline solid (amino-ethanol phosphate) formed, which was ground and dropped slowly into 13 ml. phosphorus oxychloride kept at 0°. After the vigorous reaction had subsided, the flask was removed from the ice-bath and the mixture was refluxed at 100° for 1 hour. The supernatant liquid was poured off and rejected. The gummy mass which remained was dissolved in 400 ml. water and powdered baryta added until the solution was at p_{H} 10. It was then centrifuged and the supernatant solution poured into 4 volumes of 95 % alcohol. The precipitate, after washing with alcohol and ether and drying, weighed 14.4 g. and contained 7.23 % P. The yield of primary amino-ethyl phosphate was 20 % of the total organic phosphate formed in the reaction. The rest of the organic phosphorus was present as the secondary amino-ethyl phosphate which remained in solution in the 75 % alcohol.

The barium salt was dissolved in water and the barium was precipitated with sulphuric acid. The volume was reduced and the solution was poured into 4 volumes of acetone. The amino-ethyl phosphate crystallised from the acetone solution.

Analyses.

	C %	H %	N %	P %	
Phosphoric ester from tumour	17.83	5.9	9.65	21.0	$n_D^{25} = 1.497 \pm 0.001$
Amino-ethyl phosphate (synthetic)	17.81	5.84	9.46	20.9	$n_D^{25} = 1.497 \pm 0.001$
Calculated for $\text{C}_2\text{H}_8\text{O}_4\text{NP}$	17.02	5.68	9.93	21.98	

An attempt to compare the melting-point of the phosphoric ester from tumour with that of the synthetic amino-ethyl phosphate showed that both compounds decomposed above 230° with no distinct melting-point.

Preparation of flavianates of the phosphoric ester. The natural and synthetic compounds react similarly with flavianic acid. They form salts which crystallise readily from aqueous butyl alcohol when an excess of flavianic acid is present. The salts are readily dissociated into flavianic acid and the phosphoric ester when dissolved in a hot mixture of 1 part methyl alcohol and 3 parts acetone. From such a mixture the free amino-ethyl phosphate crystallises almost quantitatively.

100 mg. of amino-ethyl phosphate were dissolved in 5 ml. of water; to this were added 400 mg. of flavianic acid (2:4-dinitronaphthol-7-sulphonic acid). 10 ml. of 95 % ethyl alcohol and 100 ml. of *n*-butyl alcohol were added to the solution, which was then filtered. The filtrate was evaporated on a boiling water-bath until crystals began to form and was then cooled in a refrigerator. The yield of the flavianate of amino-ethyl phosphate was 90 % of the calculated. After filtration the crystals were washed with butyl alcohol and dried in a vacuum desiccator over sulphuric acid.

To the flavianate, dissolved in 2 ml. of water, were added 25 ml. methyl alcohol and 75 ml. of acetone. The mixture was placed on a water-bath and boiled for a few minutes. The flavianate dissociated and the crystals of amino-ethyl phosphate which formed were filtered off, washed with acetone and dried. The yield of free amino-ethyl phosphate from the flavianate was nearly theoretical. The crystals contained about 20 % P. These crystals were re-converted to the flavianate as before, using 300 mg. of flavianic acid, and the flavianate, after crystallisation from butyl alcohol, was washed and dried.

The flavianates were analysed for nitrogen (Pregl's micro-Dumas method), phosphorus (method of King [1932]) and for flavianic acid (method of Langley and Albrecht [1935]).

Analysis of the flavianates.

	N %	P %	Flavianic acid
Phosphoric ester from tumour	9.22	6.78	69.6
Amino-ethyl phosphate (synthetic)	9.26	6.81	70.0
Calculated for the flavianate of amino-ethyl phosphate	9.23	6.81	69.1

Flavianate of phosphoric ester from tumour m.p. 223°.

Flavianate of amino-ethyl phosphate (synthetic) m.p. 225°.

Preparation of amino-ethanol flavianate. Flavianates were prepared from the bases produced by hydrolysis of the phosphoric ester from tumour and from the synthetic amino-ethyl phosphate. The natural and synthetic compounds were hydrolysed with a very active phosphatase preparation [Armstrong, 1935].

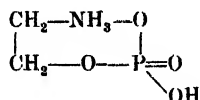
Dissolve 100 mg. of amino-ethyl phosphate in 20 ml. of water, add 0.8 ml. 0.1 *M* MgCl₂ and 10 mg. of the phosphatase preparation. Shake thoroughly and keep at 37.5°. Add slowly, with shaking, 5 ml. of a solution containing 1 part of 0.5 *N* BaCl₂ to 2 parts of 0.5 *N* Ba(OH)₂. An instantaneous hydrolysis takes place and barium phosphate is precipitated. If the hydrolysis is not complete the addition of a few mg. of additional phosphatase may be advisable or the solution may be kept for an hour or two at 37.5°. Centrifuge to remove barium phosphate, and to the centrifugate add *N* H₂SO₄ until the barium is quantitatively precipitated. Remove the barium sulphate by centrifuging. To the solution add 400 mg. of flavianic acid and 1 volume of 95 % ethyl alcohol. Reduce the volume to 20 ml., add 20 ml. of ethyl alcohol and 100 ml. of butyl alcohol. Place on a boiling water-bath and reduce the volume to about 100 ml., cool and filter. Then reduce the volume until crystals begin to form, cool in refrigerator, filter and wash the crystals with butyl alcohol and dry in a desiccator. Recrystallise by dissolving the amino-ethanol flavianate in 95 % alcohol, filter, add butyl alcohol, reduce the volume and crystallise in the refrigerator. The yield is usually about 80 % of theoretical.

	N%	M.P. (°)
Amino-ethanol flavianate (from tumour phosphate)	11.21	198
„ (from synthetic amino-ethyl phosphate)	11.18	198
„ (from pure amino-ethanol)	11.18	198
		(212)
Calculated for amino-ethanol flavianate	11.19	

When amino-ethanol flavianate was prepared from amino-ethanol, two types of crystals were obtained, one melting at 212°, the other at 198°. Usually either one type or the other was formed, but in one experiment both were formed. The crystals with melting-point 212° correspond with those described by Langley and Albrecht [1935]. The form melting at 198° was less frequently encountered when using pure amino-ethanol than when the flavianates were prepared from the products of hydrolysis. It was found that very small amounts of contaminating material cause a marked depression of the melting-point, *e.g.* an amino-ethanol flavianate preparation containing 2% of the flavianate of amino-ethyl phosphate melted at 192°. The purified amino-ethanol flavianate from the hydrolysis of amino-ethyl phosphate contained 0.4% of the flavianate of amino-ethyl phosphate (calculated from the phosphorus content) and it is conceivable that this, or some other persistent impurity, is responsible for the melting-point of 198°. The influence of the phosphatase preparation and of various solvents on the crystal form and melting-point of amino-ethanol flavianate is being studied. The fact that the crystals melting at 198° have a different refractive index from those melting at 212° suggests that two distinct crystal forms are involved and that the 198° value is not a depressed melting-point due to impurities.

DISCUSSION.

Probably the open chain formula given on p. 198 does not give the most accurate picture of the compound under discussion. There is evidence that an inner salt of the following nature:



is the form in which the compound exists in solutions at p_{H} values between 5 and 9.

This formulation is supported by the observations that: (a) the barium salt does not form, or at least cannot be precipitated from alcohol, unless the solution has a p_{H} of at least 10; (b) the brucine salt contains only one formula weight of brucine; (c) the flavianate of amino-ethyl phosphate cannot be formed from one equivalent of flavianic acid unless a p_{H} of about 3 is produced by the addition of some other acid (*e.g.* acetic) to free the amino-group from its inner salt linkage with phosphoric acid; a sufficient excess of flavianic acid will bring about the same result; (d) the brucine and flavianic acid derivatives dissociate completely on boiling in non-aqueous solvents.

Now that amino-ethyl phosphate has been identified as one of the phosphoric esters occurring in all malignant tissues studied to date, the question arises, "Does it occur in normal tissues?" At the present time the author is engaged in working up corresponding fractions from normal tissues in an attempt to isolate amino-ethyl phosphate from pancreas, liver and other organs.

SUMMARY.

One of the phosphoric esters from tumour has been shown to be identical with synthetic amino-ethyl phosphate.

The author wishes to thank Dr E. J. King, at whose suggestion the investigation of the phosphoric esters in malignant tumours was undertaken, for his encouragement and advice, and Mr C. C. Lucas for valuable criticism during the latter part of the work. The writer is also indebted to Sir F. G. Banting for his continued interest in the problem.

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XXXI. LIPOID METABOLISM IN THE MUNG BEAN DURING GERMINATION.

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(Received December 2nd, 1935.)

FAT in the seed serves presumably as a reserve food supply for the development of the young seedling. That this is especially true in the case of oily seeds has been demonstrated by many investigators. Peters [1861] early discovered that fat is transformed into carbohydrate in the germination of the pumpkin seed. Miller [1910; 1912] has shown that a successive decrease in the percentage of fat (ether extract) is accompanied by increase in carbohydrate in the development of the sunflower seedling. Recently Pierce *et al.* [1933] by studying the chemical changes and Murlin [1933] by observing the R.Q. have shown that in the castor bean fat is consumed and carbohydrate formed in the course of germination.

The lipoids in starchy seeds however have received less attention. Le Clerc and Breazeale [1911] find that the crude fat (ether extract) of wheat seedlings increases during germination. Jordan and Chibnall [1933] have studied the transfer and transformation of phosphatides and glyceride fatty acids in the germination and growth of the runner bean. Malhotra [1933] has reported the changes in energy content and reserve constituents which take place in the development of the corn seedling. In the present investigation, we have undertaken to study the changes in different reserve food constituents taking place during the germination of a typical starchy seed, *viz.* the mung bean. This has included a determination of the distribution of the lipins at each stage, and also a determination of the solid and liquid fatty acids at different stages of germination.

EXPERIMENTAL.

Preparation of material for extraction.

50 kg. of mung bean (*Phaseolus aureus*) were purchased locally. The beans were free from worms and insects.

Exactly 1 kg. was employed for each germination experiment. The beans after weighing were thoroughly washed with tap water, followed by three washings with distilled water. The washed beans were then soaked in warm distilled water at 30° for about 16 hours. During this process the beans swelled, and in many cases the seed coats were broken. The germinating sprouts were placed in metal trays in the laboratory and covered with thick wet cloth; the temperature ranged from 20 to 23°. The sprouts were washed twice daily with distilled water.

When the sprouts were 5 days old, exactly $\frac{1}{3}$ was weighed out and heated at 100° in an electric oven for 1 hour, to inactivate the enzymes. The heated sprouts were then immediately separated into cotyledons (the endosperm), germinating portion (the embryo axis), and coats (the skin). The materials in each case were then dried at a temperature of 30–35°, ground to pass a 20-mesh sieve and preserved for later extraction.

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After 10 days, the second lot was collected by weighing out exactly $\frac{1}{2}$ of the remainder; this was treated in the same manner as above. The third portion, the fraction remaining, was allowed to germinate for a total of 15 days. In this way, each lot represented exactly $\frac{1}{3}$ of the original ungerminated seeds by weight, and consequently each lot contained approximately the same number of individual beans.

The above procedure was carried through five times, and the corresponding fractions of each lot were united. It was assumed that the seed coat did not alter in composition during the germination. All the seed coats therefore were united into one single sample, weighing 510 g.

Distribution of reserve constituents during germination.

An additional 900 g. of the mung bean were sprouted, treated as above and submitted to proximate chemical analysis for all constituents. In this way, material equivalent to 300 g. of the original mung bean for each of the 5-, 10- and 15-day germination periods was obtained for analysis and is so recorded in Table I, in which the results are all stated in terms of air dried material. The

Table I. *Distribution of reserve materials in cotyledon, germinating portion and coat at various intervals during the germination of mung bean.*

(Figures in parentheses indicate totals for entire seedling at each interval exclusive of the coat.)

	Dried weight g.	Nitrogen g.	Ether extract g.	Alcoholic extract g.	Ash g.
Original seed	900.0	32.2	8.91	36.9	24.9
5 days' seedling: Cotyledon	185.5	7.27	1.95	9.55	4.29
Germinating portion	33.0	1.65	1.07	12.04	2.12
	(218.5)	(8.92)	(3.02)	(21.59)	(6.41)
10 days' seedling: Cotyledon	114.0	5.35	1.63	10.46	3.24
Germinating portion	81.5	4.42	2.49	34.63	3.90
	(195.5)	(9.77)	(4.12)	(45.09)	(7.14)
15 days' seedling: Cotyledon	50.0	2.71	1.39	8.05	1.91
Germinating portion	115.5	6.84	3.43	43.80	5.08
	(165.5)	(9.55)	(4.82)	(51.85)	(6.99)
Coat	90.5	1.59	0.63	3.88	1.96
Ungerminated seed	25.0	0.96	0.25	1.03	0.75
Total recovered	695.0	30.79	12.84	123.44	23.25

nitrogen content was determined by the usual Kjeldahl method. The extraction with ether was carried out in a Soxhlet apparatus for 24 hours, the extract being dried at 100° to constant weight, and this was followed immediately by extraction with alcohol in the same apparatus. The treatment with alcohol was continued till the extraction of the coloured substances in the sprout material was complete; this required from 30 to 72 hours. The ash was determined by igniting weighed samples to constant weight at a temperature of 400–500° in an electric furnace. The results in Table I have been recalculated to a percentage basis in Table II.

It will be noted from Table I that during germination there is a gradual transfer from the cotyledon to the germinating portion of all the constituents determined in the analysis. Under the conditions of growth imposed (absence of sunlight), the total amount of inorganic matter and of nitrogen in the plant as a whole remain practically constant for the entire 15-day period. The ether-soluble and alcohol-soluble fractions both increase in amount from stage to

Table II. *Proximate analysis of cotyledon, germinating portion and coat, at various intervals during the germination of mung bean.*

(In percentage of air-dried material.)

	Dried weight %	Nitrogen %	Ether extract %	Alcoholic extract %	Ash %
Original seed	100.0	3.54	0.99	4.10	2.77
5 days' seedling: Cotyledon	61.7	3.95	1.05	5.14	2.31
Germinating portion	11.0	5.00	3.25	36.50	6.48
10 days' seedling: Cotyledon	38.0	4.68	1.42	9.16	2.84
Germinating portion	27.2	5.43	3.06	42.51	4.82
15 days' seedling: Cotyledon	16.7	5.41	2.78	16.30	3.82
Germinating portion	38.5	5.77	2.97	37.92	4.44
Coat	10.1	1.77	0.70	4.28	2.17

stage; this seems to indicate a conversion of starch into fat and into soluble sugar. The ungerminated seeds comprised about 3% of the original seeds, and their constituents are assumed to be the same as those of the original seed.

Analysis of the ether extract (lipoid fraction).

The method of analysis followed was essentially that of Chibnall and Sahai [1931], except that dried material only was used in the ether extractions as recommended by Jordan and Chibnall [1933]. The material was in each case first powdered and extracted with ether in the Soxhlet apparatus for 24 hours: the extracted material was dried in a vacuum oven at 40°, and the material thus obtained was reported as total ether extract. The extract was then redissolved in 1 vol. of ether (50 ml. ether for each 10 g. of the extract), and 3 vols. of acetone were added. A white precipitate of phosphatides and crude hydrocarbons was always obtained. This treatment was repeated using minimum volumes of ether and acetone. The amount of precipitate obtained was so small that it did not permit of further separation by hot acetone into phosphatides, waxes *etc.*

The extract obtained by the ether-acetone treatment, containing the glyceride fats, sterols *etc.* was evaporated nearly to dryness, dissolved in ether and diluted exactly to 100 ml. A 1 or 2 ml. portion of the ethereal solution was taken for sterol determination by the digitonin method of Jowett and Lawson [1931]; 2 ml. were used for the determination of iodine value by the Hanus method. The remainder of the solution was again evaporated to dryness and the total free fatty acids obtained from this residue by the usual saponification method, which was briefly as follows: the fats were saponified by refluxing for 2 hours with 50 ml. of 5% KOH in alcohol, the alcohol evaporated and the soaps decomposed by adding 200 ml. of 3% HCl and boiling for 10 min. The free fatty acids were separated in a separating funnel, and the small portion of free fatty acid remaining in solution was extracted with ether and combined with the main portion. The fatty acids were finally washed with distilled water until free from chloride.

The results of the analysis of the ether extract are shown in Table III. An increase in total fatty acids and in the sterols is noted in successive stages. The phosphatide fraction however shows a slight decrease. It may be noted that the figures for ether extract reported in Table I differ somewhat from those in Table III. This difference is essentially due to the temperature at which the

Table III. *Analysis of ether extract of mung bean seeds and germinating sprouts.*

(Expressed in g. per kg. of the original seed.)

	Dried weight	Ether extract	Phosphatides and crude hydrocarbons (including waxes)	Total fatty acids	Sterols	I.V. of ether extract*
Original seed	1000.0	14.71	3.21	9.50	0.63	74.2
5 days' seedling:						
Cotyledon	603.2	6.15	0.23	4.52	0.44	93.6
Germinating portion	149.0	4.61	0.07	3.32	0.37	81.1
10 days' seedling:						
Cotyledon	442.0	6.21	0.12	4.50	0.48	85.0
Germinating portion	261.8	7.97	0.08	5.34	0.53	61.4
15 days' seedling:						
Cotyledon	272.1	6.22	0.14	4.28	0.52	87.7
Germinating portion	347.6	9.14	0.09	6.40	0.56	63.7
Coat	102.0	0.59	0.07	0.24	0.07	201.5

* The iodine value was determined on the ether extract after the phosphatides and crude hydrocarbons were removed.

material was dried: in the former case the extract was dried at 100° and in the latter case in a vacuum oven at 40°. The free fatty acids were submitted to further analysis as follows.

Determination of solid (saturated) and liquid (unsaturated) fatty acids in the total free fatty acids.

Twitchell's [1921] lead acetate method was used in the separation of liquid and solid soaps. Each soap fraction after being separated was decomposed into fatty acids by boiling in 3% HCl and then separated from the aqueous solution by means of a separating funnel. The material was finally washed with water until free from acid (free from chloride), and dried to constant weight in an electric oven at 40°, under reduced pressure in an atmosphere of carbon dioxide. This determination was not performed on the seed coat fraction, since the total free fatty acid obtained was insufficient. The results are shown in Table IV.

Table IV. *Solid and liquid fatty acids in mung bean seeds and germinating sprouts.*

(Expressed in g. per kg. of the original seed.)

	Total fatty acids	Solid fatty acids	Liquid fatty acids	I.V. of solid fatty acids	I.V. of liquid fatty acids
Original seed	9.50	4.38	4.97	41.2	96.2
5 days' seedling: Cotyledon	4.52	2.19	2.31	56.5	152.3
Germinating portion	3.32	1.73	1.56	61.0	127.3
10 days' seedling: Cotyledon	4.50	2.34	2.14	55.8	143.7
Germinating portion	5.34	2.44	2.72	57.6	116.2
15 days' seedling: Cotyledon	4.28	2.21	2.05	58.4	151.7
Germinating portion	6.40	2.95	3.02	57.0	112.8
Coat	0.24	—	—	—	—

RESULTS AND DISCUSSION.

We have followed the method of Jordan and Chibnall [1933] in expressing results on a weight basis taking the original seed as a unit (Table I). This arrangement emphasises the significant changes taking place during the process of germination more effectively than if expressed on a percentage basis (Table II). 100 mung bean seeds weighed 3.5 g.; this means that the figures for the analysis in Tables I and II, based on 900 g. of mung bean seeds, represent a total of 26,000 individual plants.

In the whole germinating plant, as shown in Table I, there is no change in the nitrogen and ash contents in the three stages of germination. It is significant that these constituents are carefully conserved by the plant, so that during a period of 15 days, very little was lost, even though the sprouts were washed with an abundance of water twice a day during the entire germinating period. The total dry weight of the plant decreased while the amounts of ether extract and alcoholic extract increased. This is the usual situation when carbohydrates are consumed in respiration and starch and proteins transformed into soluble amino-acids and soluble sugars. The distribution of constituents between cotyledon and germinating portion in succeeding stages demonstrates how the cotyledon functions as a storage organ for the germination of the plant. Le Clerc and Breazeale [1911] have also shown in the germination of another typical starchy seed, wheat, that the ether extract of the whole seedling is increased and the nitrogen, fibre and other constituents translocated from the seed residue into the axis.

Beumer [1933] found that sterols were synthesised during the germination of beans kept in the dark, but that they were utilised when the germination was carried out under sunlight. Our figures for the sterol fraction in Table III show a gradual increase at successive stages, which would indicate that sterols are synthesised both in cotyledon and germinating portion, when the beans are sprouted in the dark. Our observation that the phosphatide fraction is utilised by the plant during germination accords with the results of Jordan and Chibnall [1933] in experiments on the germinating runner bean.

Whilst the fat fraction in the starchy seed does not function as an energy store, there are changes in the fats and fatty acids which are significant. In the first place it is noted that the fat, whether estimated as ether extract or as total fatty acids, decreases very considerably during the first 5 days' germination, but this is followed by successive increases in the later stages of germination. It appears that in the early stages the fat fraction either serves in part as an energy reserve or takes part in a sudden tissue breakdown which accompanies the growth impulse. It is evident however that for the most part fat exists in the starchy seed as *l'élément constant* [Terroine, 1919] in contrast to *l'élément variable*. It is noted that in the cotyledon the amount of fat, evidently tissue fat, remains practically constant (Table IV), whilst an increase of fat, evidently the synthesis and the building up of tissue fat, takes place in the germinating portion. In the germination of fatty seeds, Miller [1910; 1912] has shown a similar decrease in the ether extract for the first 5 days of germination in the hypocotyl and root portion, which is followed by increases in the later periods. The i.v. determinations give further evidence of this apparent reversal from fat breakdown to fat synthesis after the first stage in germination, and, as seen in Tables III and IV, the greatest change in the i.v. takes place in the germinating portion.

Table IV shows that the transfer of liquid (unsaturated) fats from the cotyledon into the germinating portion, or their synthesis, is more rapid than that of solid (saturated) fats. The iodine values indicate a rapid increase in the degree of saturation of the liquid fat; in other words the process of germination appears to be a reducing process. The increase in degree of saturation during germination, observed in these experiments on a starchy seed, accords with the observations of Miller [1910; 1912] and of Komatsu and Okada [1933] on oily seeds (sunflower and soy bean).

The analysis of the seed coat, shown in Table III, indicates that the fatty constituents in this portion of the plant are small in amount. It is evident however that the phosphatides and crude hydrocarbon fraction of the ether extract represents a relatively more important part of the seed coat than of the other portions of the plant. A portion of this fraction was treated with hot acetone and found to be completely soluble: that is, the mixture most probably consists of waxes. These waxes apparently serve an important part in the protecting mechanism of the seed coat. It is of note that the fatty substances of the seed coat show a very high i.v.

SUMMARY.

The mung bean has been studied as an example of a starchy seed and changes in the lipoids have been examined for three 5-day intervals during germination in the absence of sunlight.

The amounts of the ethereal and alcoholic extracts of the whole mung bean sprout increase during the successive stages, whilst the total nitrogen and ash contents remain constant. At the same time, the principal constituents of the cotyledon are gradually transferred to the germinating portion of the plant.

There is an increase in the sterol fraction and a decrease in crude phosphatides during germination. The total fatty acids decrease in the early stages and increase again in the later stages. The fatty acid content of the growing portion of the plant shows a pronounced increase whilst that of the cotyledon remains practically constant. The degree of saturation of the fatty acid in the sprouting portion also shows a decided increase, as indicated by decreasing i.v.

The writer wishes to express his sincere thanks to Prof. William H. Adolph for valuable suggestions and advice throughout the progress of the work, to the China Foundation for a fellowship grant and to the Department of Chemistry of Yenching University for laboratory facilities.

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XXXII. REVERSIBLE OXIDATION OF ASCORBIC ACID BY MEANS OF NORITE CHARCOAL.

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DURING the course of some work on the excretion of ascorbic acid in urine, using the method of measurement described by Birch *et al.* [1933], difficulty was sometimes experienced in obtaining sharp end-points when dealing with highly coloured specimens. In an endeavour to overcome this recourse was had to norite charcoal, and it was then found that although the urine was successfully decolorised, it no longer reduced the indophenol. That the ascorbic acid had been converted into the reversibly oxidised form, dehydroascorbic acid, by means of the norite is shown by the following experiments.

Exp. 1. 100 ml. of filtered orange juice had a titration value of 2.3 ml. per ml. with 0.05% indophenol solution. On shaking with 2.5 g. norite and allowing to stand for about 15 min. it was found on filtering that the reducing capacity had been entirely lost.

Exp. 2. 100 ml. filtered orange juice, on treatment with 0.4 g. norite, lost part of their reducing capacity in the first 10 min.; prolonged contact with the norite did not, however, lead to any further change in the titration value.

Exp. 3. All attempts to elute the vitamin from the norite having failed, the filtrate was examined. On passing hydrogen sulphide through the solution and then clearing it by treatment with carbon dioxide for 20 min., about 90% of the original titre was regained. This property and the stability of the solution so obtained are shown in Table I.

Table I. *Oxidation and subsequent regeneration of ascorbic acid in orange juice.*

Exp.	Mixture	Treatment with norite		Regeneration of ascorbic acid		
		Titration with 0.05% indophenol		Method	Titration with 0.05% indophenol	% recovery
		Before norite treatment	After norite treatment			
1	225 ml. to 10 g. norite	3.0	0.02	Reduced with H ₂ S and excess removed with CO ₂	2.6	87
2	200 ml. to 12 g. norite	2.6	0.01	(a) H ₂ S and CO ₂ as above (b) Saturated with CO ₂ . Kept on ice 4 days then treated with H ₂ S and CO ₂ as before	2.3 2.1	89 80
3	200 ml. to 12 g. norite	2.2	—	(a) H ₂ S and CO ₂ (b) As 2 (b)—on ice 3 days	1.8 1.2	82 55
4	220 ml. to 10 g. norite	2.7	0.02	(a) H ₂ S and CO ₂ (b) As 2 (b)—on ice 2 days (c) After 14 days on ice	2.2 1.8 0.6	81 67 —

Exp. 4. The effect of varying the amount of norite used is shown in Fig. 1. It will be seen that only for small quantities is the effect directly proportional to the amount taken.

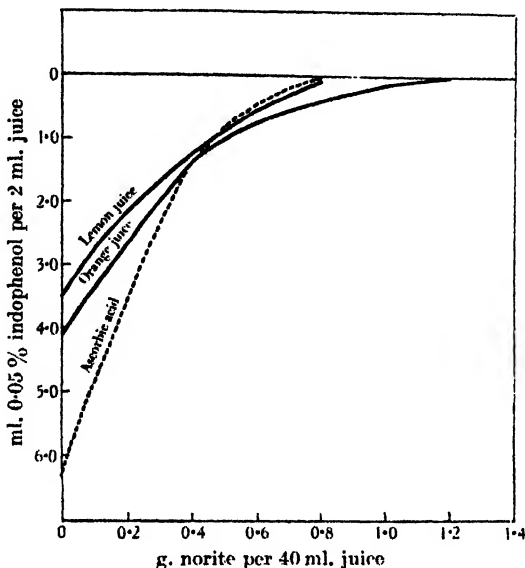


Fig. 1. Effect of varying quantities of norite charcoal on conversion of ascorbic acid into dehydroascorbic acid.

Exp. 5. The relative values of different kinds of charcoal in bringing about this reaction were examined and it was found that, whereas bone and wood charcoal had little effect, blood charcoal was fairly active, though not as good as norite.

Very little has appeared in the literature regarding the oxidising powers of charcoal suspensions, but attention is drawn to the following papers.

Feigl [1921] has shown that many substances can be oxidised by treatment with blood charcoal, either in acid or alkaline solution. Warburg and Negelein [1921] have estimated quantitatively the oxidation of cysteine and other amino-acids when placed in contact with blood charcoal. Rideal and Wright [1925] describe the oxidising action of blood charcoal on certain dicarboxylic acids, various amino-acids *etc.* and state that substances with only one polar group, *e.g.* alcohol, formic acid and the higher fatty acids, are not attacked, but that those with adjacent polar groups, one of which may be a mobile hydrogen atom, are readily oxidised. This latter observation would account for the oxidation of ascorbic acid. Finally, Fürth and Kaunitz [1929] have shown that inactivation of the charcoal occurs during the process of oxidation of amino-acids, and that addition of fresh charcoal renews the oxidation. This finding, taken in conjunction with *Exp. 4* strongly suggests that the reactive oxygen is that occluded by the charcoal.

SUMMARY.

1. Norite charcoal rapidly converts ascorbic acid into dehydroascorbic acid, with very little loss by further oxidation to the irreversible stage. The dehydro-ascorbic acid prepared in this way remains fairly stable for several days if saturated with carbon dioxide and stored on ice.

2. Since with small quantities of norite the reaction is rapid, short-lived and proportional to the amount used, it is evidently not of the same type as that brought about by traces of iron or copper [Kellie and Zilva, 1935; Mawson, 1935]. Most probably it is a case of direct transference of oxygen occluded on the surface of the charcoal.

3. Attention is drawn to the simplicity of this reaction and its value where mild oxidation is required, unaccompanied by undesirable by-products.

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XXXIII. EXPERIMENTS CONFIRMING THE ANTISCORBUTIC ACTIVITY OF DEHYDROASCORBIC ACID AND A STUDY OF ITS STORAGE AND THAT OF ASCORBIC ACID BY THE GUINEA-PIG AT DIFFERENT LEVELS OF INTAKE.

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HIRST AND ZILVA [1933] showed that the antiscorbutic value of dehydroascorbic acid prepared by oxidation of ascorbic acid with iodine was only slightly less than that of the ascorbic acid from which it had been prepared. Having devised a simple method for preparing dehydroascorbic acid free from contaminating substances [Fox and Levy, 1936] it was thought worth while to study this interesting observation in greater detail. At the same time advantage was taken of the chemical method now available for estimating ascorbic acid to try to learn something about the metabolism of these two substances at different levels of intake.

Exp. 1. 12 young guinea-pigs were placed on the scurvy-producing diet described by Bracewell *et al.* [1930]. The progress of the four groups into which these animals were divided is charted in Fig. 1.

The norite-orange juice (N.O.J.) was prepared by treating 250 ml. fresh juice with 10 g. of norite and allowing to stand for 15 min.; the liquid was then filtered through a thick layer of cotton-wool and stored in the ice-chest until required. No attempt was made to remove the last traces of norite. The N.O.J. was prepared every second day or rarely every third day during the course of the experiment, each batch being tested before use by means of the indophenol reagent and shown to be entirely free from reducing substance.

All the animals on the dehydroascorbic acid ration when examined *post mortem* were found to be entirely free from any signs of scurvy. It was, however, noticed that the adrenals of animals 9, 10, 12, 12 (b) and 12 (c) looked somewhat flabby and wrinkled. On microscopic examination it was found that each of these cases showed evidence in varying degrees of fatty change affecting the outer cortical zone, most marked just underneath the capsule. The majority of the cells resembled those found in xanthomatous degeneration, *i.e.* they were converted into foamy cells. In addition many of the cells were distended with definite large droplets of fat. Whilst the medulla invariably showed no abnormality, the cortex was in each case the seat of marked congestion.

Conclusion. This experiment confirms the observation that dehydroascorbic acid possesses antiscorbutic activity. Not only was it able to prevent the appearance of scurvy over a period of 14 weeks, but 2 animals gave birth to live young whilst on the diet and one of the young was successfully reared on the oxidised vitamin. It is not clear what significance can be attached to the changes observed in the adrenals.

In addition to the clinical evidence regarding freedom from scurvy obtained above it was decided to estimate the amount of antiscorbutic vitamin in the livers and adrenals of all the animals included in the experiment. For this purpose we used the method described by Birch *et al.* [1933].

Exp. 2. With the object of first finding out what the normal level of ascorbic acid in these organs might be we examined some animals that had been killed to provide complement for the Wassermann reaction. They had all been living on a liberal diet of cabbage and bran. As will

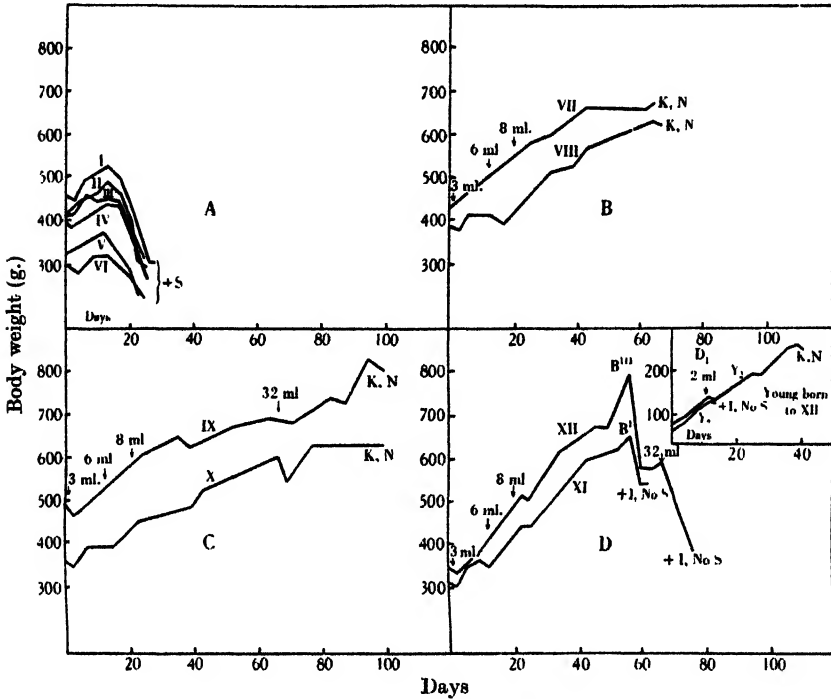


Fig. 1. Growth curves of guinea-pigs on a basal diet *plus* dehydroascorbic acid. A. Negative controls. Basal diet only; all animals died within 23–28 days, showing in each case well marked evidence of scurvy. B. Basal diet *plus* norite-orange juice (dehydroascorbic acid). Animals remained in excellent condition and showed no signs of scurvy when killed after 64 days. C. Similar diet to B but larger quantities of N.O.J. given after 64th day. No evidence of scurvy when killed after 100 days. D. Similar diet to B. 4 young born during experiment (64th day). Both animals died several days later from intercurrent infection, but showed no evidence of scurvy. D₁. Of the 4 young, 2 died within a day after birth; the other 2 were separated from the mother (guinea-pig 12) at the end of a week and were fed on the basal diet *plus* N.O.J. 1 remained alive for 2 weeks, whilst the other continued to grow and was in excellent condition when killed after having been reared for 40 days on the N.O.J. *Post mortem*, both these animals were found to be completely free from scurvy.

K = Killed.
N = Normal.
I = Intercurrent infection.
+ = Died.
No S = No Scurvy.

S = Scurvy.
B₁ = Birth of 1 young.
B₁₁ = Birth of 3 young.
↓ = Amounts of norite-orange juice given daily.

be seen from Table I (a) very fairly constant values were obtained for the 8 animals examined. These results are in good agreement with those recorded by Svirbely [1933], whose mean values for 4 animals fed on a basal diet *plus* excess of spinach were 0.23 and 1.24 mg. per g. for liver and adrenals respectively. Similarly Zilva [1935] obtained 0.25 mg. per g. of liver as an average for 4 animals on a mixed diet.

Substantially the same values were again obtained when the above diet was supplemented with 4–6 ml. of orange juice daily (Table I (b)).

Those animals were all killed about 20 hours after their last feed of cabbage. Table I (c) shows that substantially higher concentrations were obtained when the animals had free access to cabbage until just before they were killed.

Table I. *Ascorbic acid content of liver and adrenals of normal guinea-pigs placed on various diets.*

Animal	Liver		Adrenals	
	Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.
(a) On a diet of cabbage and bran.				
1	19.0	0.26	0.160	1.70
2	17.5	0.17	0.346	1.30
3	17.9	0.21	0.500	1.24
4	33.5	0.31	0.852	1.48
5	19.3	0.32	0.390	1.23
6	20.0	0.23	0.722	1.10
7	18.8	0.28	0.293	1.62
8	24.8	0.23	0.440	1.62
9	15.4	0.24	0.165	1.68
10	23.6	0.20	0.181	2.06
Mean	21.0	0.25	0.405	1.50
(b) On the same diet plus 4-6 ml. orange juice per day.				
11	20.0	0.25	0.132	1.48
12	31.0	0.31	0.360	1.33
13	22.5	0.33	0.153	1.28
Mean	24.5	0.30	0.215	1.36
(c) On same diet, but with free access to cabbage until killed.				
14	20.9	0.52	0.120	2.14
15	25.6	0.45	0.185	2.02
(d) On same diet plus 100 mg. ascorbic acid per day for 8 days and with free access to cabbage until killed.				
16	18.0	0.42	0.147	2.06
17	25.8	0.38	0.153	1.96

In order to convince ourselves that this new level represented the maximum concentration obtainable, two more animals were given the same diet plus 100 mg. of ascorbic acid (redoxon) daily; this dose of more than 100 times the minimum protective dose was continued for 8 days and the animals were then killed after having access to cabbage overnight. Table I (d) shows that the concentration previously obtained was not exceeded; presumably this represents the saturation point for the liver of a guinea-pig.

Conclusion. From these experiments it is clear that the guinea-pig has a small and definite capacity for storing the vitamin in its liver; this maximum value can easily be reached on an ordinary herbivorous diet.

Exp. 3. In order to form some more definite idea as to the normal intake of ascorbic acid 12 guinea-pigs of about 350 g. weight were placed on a diet of bran plus cabbage *ad lib.* The weights of cabbage consumed on 4 successive days were found to be 240, 410, 370 and 330 g. giving an average consumption of about 30 g. per animal. Assuming a value of 0.4-0.6 mg. ascorbic acid per g. this would represent an intake of from 12 to 18 mg. *per diem*, or between 10 and 20 times the minimum protective dose. This definite but rather limited power of storage no doubt accounts for the well-known fact that the onset of scurvy in guinea-pigs cannot be delayed to any extent by the previous administration of large doses of the vitamin [see for example *Medical Research Council Report on Vitamins*, 1932].

Exp. 4. The amounts of ascorbic acid found in the animals used in Exp. 1 are recorded in Tables II and III.

It will be seen that animals dying from scurvy showed very low stores of the vitamin and indeed it is doubtful whether the very small titration obtained was due to ascorbic acid at all. This finding is again in agreement with those recorded by Svirbely [1933] for similar animals dying from scurvy [*cf.* Hamilton, 1933].

Table II. *Ascorbic acid contents of liver and adrenals of guinea-pigs dying from scurvy.*

Animal	Days on diet	Liver		Adrenals	
		Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.
1	28	10.0	0.014	0.44	≤ 0.024
2	25	13.2	0.016	0.60	≤ 0.017
3	25	9.7	0.021	0.44	≤ 0.023
4	26	15.0	0.018	0.34	≤ 0.030
5	23	11.3	0.025	0.38	≤ 0.026
	Mean	11.8	0.019	0.44	≤ 0.024
Animal treated with N.O.J. 2 days prior to death.					
6	25	12.4	0.064	0.50	0.21

Table III. *Ascorbic acid contents of liver and adrenals of guinea-pigs fed on norite-orange juice.*

Animal	Age in days	Days on N.O.J.	Dose per day ml.	Liver		Adrenals	
				Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.
7	—	64	8	32.7	0.021	0.41	0.10
8	—	66	8	29.7	0.065	0.15	0.56
9	—	100	32	34.2	0.100	0.49	0.80
10	—	99	32	23.5	0.120	0.35	0.70
11	—	62	8	29.0	0.038	0.41	≤ 0.03
12	—	77	8	30.3	0.066	0.52	0.13
		Mean		29.9	0.068	0.39	0.39
Young born during experiment.							
11 (a)	1	—	—	4.1	0.06	0.021	≤ 0.48
12 (a)	1	—	—	2.7	0.08	0.033	≤ 0.30
12 (b)	16	9	4	5.7	0.07	0.060	0.50
12 (c)	42	35	8	12.6	0.08	0.100	0.74

It was the quite unexpected finding that animals 7 and 8, although receiving plenty of N.O.J. and with no signs of scurvy, had such low reserves of vitamin that led us to feed the remaining animals for a longer time and on very generous quantities of dehydroascorbic acid. As will be seen, however, it made little difference, for the stores of animals 9 and 10 were very nearly as low as the preceding ones. Similar low results were also obtained with animals 11 and 12. Finally, the 4 young born during the experiment, including the animal which remained in excellent health until killed when 6 weeks old, also showed the same low reserve of vitamin C, yet were entirely free from clinical evidence of scurvy.

DISCUSSION.

In seeking an explanation for these surprisingly low reserves the following possibilities call for consideration.

(1) That the animals cannot have been living just above the scurvy border line owing to traces of ascorbic acid present in the basal diet is evident from the fate which so rapidly overtook the six animals that were on this diet alone.

(2) That similar traces of ascorbic acid were supplied by the norite-orange juice is also most unlikely since repeated controls showed that it had no action whatever on indophenol.

(3) That the liver was not storing the vitamin as dehydroascorbic acid was shown by the fact that preliminary treatment of the liver extract with hydrogen sulphide did not increase the titre.

(4) We therefore concluded that dehydroascorbic acid, though effective as an antiscorbutic, was not as easily stored as the parent substance.

With the hope of throwing more light on the ability of the guinea-pig to store ascorbic acid at different levels of intake three further experiments have been carried out.

Exp. 5. In order to determine the lowest level of storage that the guinea-pig can attain without clinical evidence of scurvy 5 animals were placed on the usual basal diet and killed after 10 days, *i.e.* at the time when the weight usually begins to fall [see also Siehrs and Miller, 1933; 1934]. Table IV shows that by this time the reserves are almost completely exhausted although no clinical evidence of scurvy was obtained.

Table IV. *Ascorbic acid contents of the liver and adrenals of guinea-pigs killed after 10 days on the scorbutic diet.*

Animal	Liver		Adrenals		Remarks <i>Post mortem</i> examination
	Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.	
1	13.4	0.037	0.34	0.24	No scurvy
2	13.7	0.017	0.18	0.11	Slight scurvy
3	19.8	0.028	0.26	0.19	No scurvy
4	22.0	0.017	0.39	0.26	No scurvy
5	21.0	0.030	0.23	0.22	No scurvy
Mean	18.0	0.026	0.28	0.20	

Exp. 6. In this experiment 4 guinea-pigs were placed on the basal diet *plus* a daily ration of 5 ml. of orange juice (equivalent to 2.5–3.0 mg. ascorbic acid as found by titration). On this amount, which was intermediate between the minimum protective dose and that present in a diet of unrestricted cabbage or similar food, it was found that the storage was again of a low order (Table V). As this diet had been continued for 2 months it is clear that had there been but a slight storage of the available vitamin day by day it would have soon led to quite a considerable reserve [*cf.* Hamilton, 1933].

Table V. *Ascorbic acid contents of the livers and adrenals of guinea-pigs fed for 2 months on basal diet plus 5 ml. orange juice (\equiv 2.5–3.0 mg. ascorbic acid per day).*

Animal	Liver		Adrenals	
	Weight g.	Weight acid per g. mg.	Weight g.	Weight acid per g. mg.
1	21.8	0.063	0.28	0.41
2	25.2	0.104	0.24	0.58
3	22.0	0.054	0.21	0.51
4	25.6	0.073	0.33	0.48
Mean	23.6	0.073	0.27	0.50

Exp. 7. Further confirmation of the results of the foregoing experiment was obtained by supplying varying amounts of ascorbic acid to guinea-pigs, this time, however, in the form of lucerne leaves. The animals were on the same basal diet and were given weighed quantities of the leaves, the ascorbic acid content of which had been estimated previously by means of indophenol. The feeding was continued for nearly 100 days [see, for further particulars, Levy and Fox, 1935]. Here again it will be noticed (Table VI) that although the animals were fully protected from

Table VI. *Ascorbic acid contents of the livers and adrenals of guinea-pigs fed for 3 months on a basal diet plus lucerne leaves.*

(Average value of lucerne leaf, 1 g. = 2.67 mg. ascorbic acid.)

	Liver		Adrenals		
Animal	Weight g.	Ascorbic acid per g. mg.	Weight g.	Ascorbic acid per g. mg.	Remarks
(a) Controls (basal diet only).					
1	8.6	0.043	0.32	0.057	Died of scurvy
2	9.4	0.041	0.44	≤ 0.027	"
Mean	9.0	0.042	0.38	0.042	
(b) Basal diet <i>plus</i> 0.8 mg. ascorbic acid per day.					
3	25.1	0.044	0.32	0.22	Killed
4	19.4	0.042	0.50	0.09	Died of intercurrent infection
5	27.4	0.030	0.40	0.13	Killed
6	45.3	0.022	0.55	0.14	"
7	29.7	0.025	0.63	0.15	"
Mean	29.4	0.033	0.48	0.15	
(c) Basal diet <i>plus</i> 1.6 mg. ascorbic acid per day.					
8	25.8	0.10	0.45	0.13	
9	17.1	0.12	0.28	0.17	Both animals died of intercurrent infection
Mean	21.5	0.11	0.37	0.15	
(d) Basal diet <i>plus</i> 3.2 mg. ascorbic acid.					
10	20.3	0.062	0.27	0.45	Killed
11	27.1	0.054	0.28	0.37	"
12	30.9	0.053	0.42	0.28	"
13	40.7	0.052	0.33	0.21	"
14	29.1	0.043	0.25	0.20	"
Mean	29.6	0.053	0.31	0.32	

macroscopic evidence of scurvy, even when fed on very small quantities of the lucerne at these lower levels of intake of ascorbic acid, they were unable to build up any considerable reserve of the vitamin. It is also of interest to notice that the amounts found in liver and adrenals are closely similar to those obtained by feeding the same quantity of ascorbic acid contained in orange juice. This affords an interesting proof of the availability of the vitamin in green leaves as compared with a fruit juice.

SUMMARY.

1. The antiscorbutic value of dehydroascorbic acid has been fully confirmed in experiments with guinea-pigs lasting over a period of 14 weeks. One animal, born during the course of the experiment, was successfully reared on the oxidised vitamin.

2. Dehydroascorbic acid was reduced to ascorbic acid and stored to a small extent in the liver of these animals; the amount stored remained low, even when large doses were given over a considerable period.

3. On the other hand it is seen that with ascorbic acid the amount stored is closely dependent upon the actual level of intake. At levels little greater than the minimum protective dose hardly any storage takes place; at intermediate levels the storage is approximately proportional to the intake, whilst with larger amounts an upper limit soon appears to be reached.

How far these findings apply to the human being remains to be seen, but they are in general agreement with the conclusions arrived at by Johnson and Zilva [1934] and by Harris and Ray [1935] based on urinary excretion tests.

We are indebted to Dr F. W. Simpson, of the Pathological Department of the Institute, for his help with the *post mortem* examination of the animals and for preparing and examining the sections of the adrenals; also to Messrs Hoffman La Roche for the gift of ascorbic acid.

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XXXIV. NOTE ON THE NON-IDENTITY OF LACTOFLAVIN AND THE "EXTRINSIC FACTOR" IN PERNICIOUS ANAEMIA.

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(Received December 19th, 1935.)

THE maintenance of a normal red cell count in human blood is apparently dependent on the presence in the gastric secretion of an "intrinsic factor"—haemopoietin—which is of the nature of an enzyme [Castle *et al.*, 1929; 1930; Klein and Wilkinson, 1933; 1934; Wilkinson and Klein, 1932; 1933].

A deficiency of this factor in the gastric secretion is closely associated with the development of the megalocytic anaemia—pernicious anaemia. Under the influence of this haemopoietin some as yet unknown constituent of the diet—the so-called "extrinsic factor"—is converted into the substance, probably identical with the anti-anaemic liver principle, which is essential for normal erythropoiesis in the bone-marrow. The nature of this "extrinsic factor" has naturally been a source of much speculation. Strauss and Castle [1932], noting the similarity of distribution of the vitamin B₂ complex and the extrinsic factor, carried out experiments using both autolysed yeast and an 80 % alcohol extract of autolysed yeast as substrates and put forward the suggestion that "the extrinsic factor may now be defined as a substance closely related to vitamin B₂, if not vitamin B₂ itself".

Since then a considerable amount of research has been directed to the examination of this point: it may be said at once that practically all the results reported fail to support this suggestion, and hence the literature requires only brief review. Wills and Naish [1933], using an egg-white extract rich in vitamin B₂ as substrate, were unable to obtain a remission when this was fed after incubation with normal gastric juice to a patient with pernicious anaemia. Lassen and Lassen [1934], using various yeast extracts, rich in vitamin B₂ and sometimes also in vitamin B₁, concluded that the extrinsic factor was not identical with vitamin B₁ or B₂. Diehl and Kühnau [1933], using a purified source of vitamin B₂, concluded that the two factors were distinct. On the other hand Miller and Rhoads [1934] obtained contrary results to Wills and Naish when using egg-white containing vitamin B₂ and also when using rice polishings concentrate as substrate. Their results, however, are somewhat equivocal as to the identity of vitamin B₂ with the extrinsic factor, since the responses obtained with the egg albumin with a high vitamin B₂ content (20 rat units per day) were poor whilst the responses with the rice polishings containing less than half that amount of vitamin B₂ were very good. They concluded that "if vitamin B₂ is the extrinsic factor it must be effective in exceedingly low concentration", and again—"conclusive proof of the identity of the dietary anti-anaemic factor and vitamin B₂ must be deferred until the isolation of the vitamin in a pure form has been effected". The reason for re-opening the question here is that the researches of György *et al.* [1934] and György [1935, 1, 2] have provided very strong evidence

of the identity of one growth-promoting constituent of vitamin B₂ with lactoflavin.¹ This substance has been placed at our disposal in quantity through the kindness of Messrs Bayers, Ltd., and this has made it possible for us to reinvestigate the point. The results though negative are considered of sufficient importance to be reported here.²

EXPERIMENTAL.

The clinical technique employed in the following experiments to detect haemopoietic activity has been fully described in previous communications. It is only necessary to emphasise here the necessity firstly for careful choice and adequate control of the test cases of pernicious anaemia used [Wilkinson and Klein, 1934], and secondly for a correct interpretation of the haematological response.

The products have been administered parenterally, since it is well established that active liver extracts are much more effective when administered intramuscularly than when given orally. Klein and Wilkinson [1934] showed that, following incubation of beef and fraction P 5 (*i.e.* the active fraction prepared by alcoholic precipitation of the press juice from hog's stomach [Klein and Wilkinson, 1933]), a protein-free substance can be prepared which is therapeutically active in pernicious anaemia and is suitable for administration by intramuscular injection. A similar technique has been employed in the experiments to be reported here.

The lactoflavin used was supplied by Messrs Bayer Products, Ltd., in ampoules each containing 2 ml. of an aqueous solution of 1 mg. lactoflavin.

The gastric juice was obtained from normal human subjects after histamine injection, only samples containing adequate amounts of free hydrochloric acid and pepsin being used.

Exp. I. Effect of lactoflavin alone. Lactoflavin solution (32 ml. = 16 mg.) was injected intramuscularly into case P.A./538 over a period of several days. There was no haematological response even after 9 days but the patient subsequently responded normally to a known active parenteral liver preparation (Table I). It will be seen indeed that the patient's condition was steadily relapsing during the injection of lactoflavin.

Exp. II. The effect of incubating lactoflavin with fraction P 5. Fraction P 5 (10 g.) was dissolved in 100 ml. of cold N/10 HCl, diluted to 400 ml. with distilled water and brought to p_{H} 4.3 by addition of N NaOH. Lactoflavin solution (32 ml. = 16 mg.) was then added and the mixture warmed to 37° and incubated at that temperature for 5 hours. The product was adjusted to p_{H} 6.0 and 2 volumes of 90 % alcohol were added. After standing for 3 days, the mixture was filtered and the alcoholic filtrate concentrated *in vacuo* to one-tenth of the volume. Two volumes of 90 % alcohol were then added, and after standing overnight the precipitate was removed and the filtrate again concentrated to small volume. After standing in the ice-chest overnight the precipitate which settled was filtered off and the filtrate reduced to a syrup *in vacuo*. Ten volumes of absolute alcohol were then added and the precipitate so obtained after standing was collected, washed with absolute alcohol and dried in a vacuum desiccator. The yield of the product (S.I. 22) was 1.5 g. Parenteral administration of this fraction

¹ György [1935, 1] regards "vitamin B₂" as a complex containing lactoflavin and vitamin B₂ [see also Chick *et al.*, 1935].

² Since carrying out our experiments, the important results of Groen [1935] have come to our notice. He obtained negative results when 5 mg. lactoflavin, incubated with 150 ml. normal gastric juice, were administered each day orally for 10 days. His previous experiments using different sources of vitamin B₂ pointed in the same direction.

Table I. *Showing lack of response in case P.A./538 to injection of lactoflavin and subsequent response to injection of an active liver extract.*

Day	Reticulocytes %	Red blood cells per μl.	Haemo- globin %	Treatment
1	1.9	1,500,000	45	} Control period. No treatment
2	—	—	—	
3	1.6	—	—	
4	1.7	—	—	
5	—	—	—	
6	1.7	1,552,000	45	2 ml. }
7	1.8	—	—	6 ml. }
8	1.7	—	—	6 ml. }
9	1.5	—	—	6 ml. }
10	1.6	—	—	6 ml. }
11	1.0	—	—	6 ml. }
12	—	—	—	Nil
13	1.4	1,176,000	36	2 ml. }
14	1.3	—	—	4 ml. }
15	1.6	—	—	6 ml. }
16	4.1	—	—	Nil
17	10.6	—	—	Nil
18	23.0	—	—	Nil
19	30.8	—	—	Nil
20	37.5	1,520,000	48	Nil
21	26.2	—	—	Nil
29	5.1	2,960,000	70	Nil
34	1.6	3,200,000	74	Nil
41	0.8	4,064,000	90	Nil

Table II. *Showing lack of response in case P.A./553 to injection of fraction S.I. 22 and subsequent response to injection of an active liver extract.*

Day	Reticulocytes %	Red blood cells per μl.	Haemo- globin %	Treatment
1	1.5	1,340,000	36	} Control period. No treatment
2	1.8	—	—	
3	2.2	—	—	
4	2.3	—	—	
5	1.7	1,250,000	34	
6	1.1	—	—	0.2 g. }
7	2.8	—	—	0.4 g. }
8	1.2	—	—	0.8 g. }
9	2.1	1,110,000	32	Nil
10	2.0	—	—	Nil
11	1.3	—	—	Nil
12	1.1	890,000	26	4 ml. }
13	1.3	840,000	26	4 ml. }
14	—	—	—	4 ml. }
15	2.6	830,000	24	Nil
16	6.1	—	—	Nil
17	24.4	880,000	—	Nil
18	16.9	—	—	Nil
19	—	—	—	Nil
20	21.7	1,120,000	27	Nil
21	17.6	—	—	Nil
22	—	—	—	Nil
23	16.1	1,320,000	34	Nil
24	13.6	—	—	Nil
25	9.8	—	—	Nil
26	7.2	—	—	Nil
27	5.1	—	—	Nil
28	—	—	—	Nil
29	3.6	1,740,000	40	Nil

to case P.A./553 during 3 days produced no clinical response after 8 days, although normal responses were obtained subsequently using a known active preparation (Table II).

The negative result obtained might have been due to an insufficient amount of substrate since the fraction P 5 used had been clinically tested and found to be potent. A further experiment using larger quantities of lactoflavin and normal human gastric juice instead of fraction P 5 (Exp. III) confirms the findings of Exp. II.

Exp. III. The effect of lactoflavin after incubation with normal gastric juice. A mixture of 78 ml. (=39 mg.) of lactoflavin solution and 1940 ml. of normal fresh gastric juice was adjusted to p_H 3.0 by addition of *N* NaOH and incubated for 3 hours at 37°. The p_H was adjusted to about 6.0 and after heating at 60–65° for half an hour to inactivate the enzymes, 2 volumes of 90 % alcohol were added. After standing for 3 days, the mixture was filtered and the alcoholic filtrate worked up as described in Exp. II. The yield of this fraction (G.I. 2) was 10 g.

The administration of this fraction intramuscularly failed to produce any haemopoietic response. Thus in case P.A./365 when 10 g. were given over a period of 4 days no clinical response was noted after 23 days, although a normal response was subsequently obtained following the administration of a known active preparation (Table III).

Table III. *Showing lack of response of case P.A./365 to fraction G.I. 2 and subsequent response to injection of an active liver extract.*

Day	Reticulocytes %	Red blood cells per μl.	Hæmo- globin %	Treatment
1	0.4	1,890,000	54	{ Control period. No treatment. Reticu- locytes below 2.5 %
7	1.3	1,775,000	52	
14	2.3	—	—	{ 1 g. 2 g. 3 g. 4 g. } Fraction (G.I. 2
15	1.9	1,680,000	50	
16	2.1	—	—	
17	1.9	—	—	
18	0.9	1,440,000	43	Nil
19	1.7	—	—	
21	0.9	—	—	
23	0.6	—	—	
25	0.7	1,200,000	36	{ 4 ml. 4 ml. 4 ml. } Intramuscular liver extract
26	0.7	—	—	
28	1.3	—	—	
37	1.1	1,260,000	38	
38	1.0	—	—	Nil
39	1.9	—	—	
40	1.2	—	—	"
41	4.2	—	—	
42	18.3	—	—	"
43	17.7	—	—	
44	19.6	—	—	"
45	7.9	2,056,000	52	
46	5.1	—	—	"
47	2.1	—	—	
52	1.8	2,820,000	64	"
59	1.0	3,320,000	82	

DISCUSSION.

The illustrative experiments reported here make it clear that lactoflavin is neither the anti-anaemic liver principle nor the "extrinsic factor" of Castle. In connection with the latter finding it should be noted, however, that Castle

himself (quoted by Lassen and Lassen [1934] and Davidson [1933]) is "prepared to relinquish the suggestion that vitamin B₂ and the extrinsic factor are identical".

The exact nature of the "extrinsic factor" or factors is as yet unknown, and its distribution among dietary foodstuffs has led to conflicting results among different workers. Yeast and yeast products have been much investigated from this point of view but there is still no satisfactory evidence as to whether the active principle in certain autolysed yeast products is solely of the nature of "extrinsic factor" or a substance similar to the anti-anaemic liver principle or even a mixture of the two produced by autolysis. The literature on the subject is full of contradictions and quite uncritical experimentation. It is clear that the methods of testing haemopoietic activity and the interpretation of the findings are still not fully understood by some workers and, indeed, cases other than those of typical uncomplicated pernicious anaemia have sometimes been employed for test purposes. In fact the whole problem of the nature of the extrinsic factor, and of the nature of the active principle in autolysed yeast preparations, needs careful and critical reinvestigation.

It appears certain on the one hand that autolysis of yeast yields a substance capable of curing certain tropical megalocytic anaemias where there is little or no gastric impairment [Wills, 1931]. This autolysate or its extracts can apparently be digested with haemopoietin, or "intrinsic factor", and the digest employed to initiate remissions in pernicious anaemia [Groen, 1935; Ungley, 1933]. Yeast preparations without preliminary autolysis are apparently inactive. It is less clear, on the other hand, whether autolysed yeast products can really be used satisfactorily to treat typical cases of pernicious anaemia. Occasional success has been described [Davidson, 1931; 1933; Goodall, 1932; Russell, 1934; Ungley, 1933; Ungley and James, 1934] but it is evident that in the published series of cases, very few have responded to this treatment, and Davidson [1933] has concluded that marmite is not in any way an efficient or reliable substitute for stomach or liver preparations.

It will suffice therefore to remark that the occurrence of the extrinsic factor in beef muscle protein has been amply demonstrated by Castle *et al.* [1929; 1930] and by Klein and Wilkinson [1934] and this material furnishes a very suitable source for testing digestion methods in conjunction with normal gastric juice or hog's stomach fractions. The possibility of the extrinsic factor being related to the glycoproteins is suggested by the recent work of Dakin and West [1935], and is also being investigated in this laboratory. It will be recalled that Reimann [1931; Reimann and Fritsch, 1934] has shown that the potency of fresh liver is much increased by incubation with normal gastric juice, presumably owing to a high content of extrinsic factor in fresh liver, and more particularly, in the liver proteins. On the other hand, the report of Walden and Clowes [1931] and of Helmer *et al.* [1932], that the potency of liver extract (Lilly No. 343) is increased by incubation with normal gastric juice is difficult to explain and obviously requires reinvestigation. (The theory that vitamin B₂ was the extrinsic factor afforded an obvious and easy explanation of this observation since liver extract is a rich source of the vitamin.) Of a different nature is the claim of Herron and McEllroy [1933] that simple autolysis for 10 days enhances the haemopoietic potency of liver. This claim is not justified by their published results and has since been disproved by Castle and Strauss [1935].

One further point requires clarification. According to György [1935, 1, 2] the lactoflavin of the vitamin B₂ complex is concerned largely with its growth-promoting properties and has no well-marked "antipellagra" or "antidermatitis"

properties for rats. The nature of the antipellagra factor is at present unknown. It is therefore of importance in this connection that Spies *et al.* [1934] have concluded that the "extrinsic factor" and the "antipellagra" factor for human subjects are distinct, although both may be contained in yeast. [See also Spies, 1935.] On the other hand several observers, *e.g.* Ramsdell and Magness [1933] and Spies [1933], have found parenteral liver extract very efficacious in the treatment of human pellagra.

SUMMARY.

The growth-promoting constituent of vitamin B₂ in the form of pure lactoflavin has been shown to be neither the liver anti-pernicious anaemia principle nor the "extrinsic factor" concerned in haemopoiesis.

We wish to record our grateful thanks to Dr L. J. Harris for his interest and assistance in obtaining the supplies of lactoflavin.

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XXXV. STUDIES IN TISSUE METABOLISM.

VIII. THE EFFECT OF FUMARATE AND SUCCINATE ON TUMOUR RESPIRATION.

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(Received December 21st, 1935.)

SZENT-GYÖRGYI [1935] has recently emphasised the importance of dibasic acids containing four carbon atoms in the respiration of muscle. He suggests that the reaction fumarate \rightleftharpoons oxaloacetate is an essential part of a chain of reactions which brings about the oxidation of foodstuffs in tissues. Oxaloacetate is in part reduced to succinate, which is rapidly converted into fumarate by the powerful succinic dehydrogenase. In the presence of malonate the reaction succinate \rightarrow fumarate is inhibited so that the fumarate disappears; the rate of respiration is correspondingly reduced. In most respects Szent-Györgyi's observations on washed pigeon muscle have been repeated upon the mammalian tumours which we have examined. Malignant tissue therefore seems to resemble muscle, kidney and liver in using C_4 dibasic acids as oxygen carriers.

EXPERIMENTAL.

The tumours used were the Jensen rat sarcoma (JRS) and Crocker mouse sarcoma 180. The tissue was sliced by Warburg's method and, in the case of "minced tissue", the slices were minced with scissors to an average size $<0.5 \times 1 \times 1$ mm. 0.2–0.3 g. moist tissue was placed in the Warburg vessels with 2 ml. *M*/15 phosphate (p_H 7.3) containing, on occasion, glucose or lactate. After 30 min. measured respiration substrates were tipped in (as solutions of Na salts) from the side arms and measurement continued for 1 hour. Q_{O_2} during this hour was expressed as a percentage of Q_{O_2} during the first 30 min. In calculating Q_{O_2} values the dry weight was taken as 1/10 wet weight.

Respiration was increased by addition of succinate or fumarate and decreased by addition of malonate (Table I), but these effects were greater with minced

Table I. *The effects of succinate, fumarate, malate and malonate on oxygen uptake in the absence of other substrates.*

Tissue	Substrates	Initial Q_{O_2}		% of initial Q_{O_2}	
		Slices	Minced tissue	Slices	Minced tissue
Crocker 180 (Exp. I)	—	6.6	6.2	38	53
"	Succinate 4 mg.	6.2	6.7	40	77
Crocker 180 (Exp. II)	—	—	5.7	—	72
"	Succinate 4 mg.	—	5.7	—	95
"	Fumarate 4 mg.	—	6.2	—	82
"	Malonate 2 mg.	—	5.8	—	48
"	Malonate 2 mg. + fumarate 4 mg.	—	5.4	—	71
JRS	—	10.4	10.3	77	70
"	Succinate 4 mg.	9.4	10.1	91	109
"	Fumarate 4 mg.	10.6	9.8	86	89
"	Malate 3.5 mg.	10.2	10.5	80	82
"	Malonate 2 mg.	10.2	10.2	53	40
"	Malonate 2 mg. + fumarate 4 mg.	10.4	9.6	74	69

than with whole slices. The effects were observed irrespective of the presence of glucose or lactate (Table II). This agrees with the suggestion that the C_4 dibasic acids function as oxygen carriers.

Table II. *The effects of succinate, fumarate and malonate on oxygen uptake in presence of glucose or lactate.*

(a) Minced JRS, (b) minced JRS with 4 mg. glucose,
(c) minced Crocker 180 with 4 mg. lactate.

Substrates	Initial Q_{O_2}			% of initial Q_{O_2}		
	a	b	c	a	b	c
---	7.8	8.3	5.8	77	90	62
Succinate 4 mg.	7.6	8.1	6.0	102	112	105
Fumarate 4 mg.	8.2	7.9	6.2	84	98	78
Malonate 2 mg.	7.9	8.0	—	61	67	—
Malonate 2 mg. + fumarate 4 mg.	7.8	8.1	—	74	85	—

In experiments using fumarate and malonate, it was found that though fumarate might counteract the inhibitory effect of malonate by stimulating respiration up to the control value, this value was not exceeded, whereas alone it usually increased the oxygen uptake above that of the control. Since this might be due to the use of insufficient fumarate, experiments were repeated at higher concentrations of fumarate. Table III shows that relatively high fumarate con-

Table III. *The effects of varying fumarate concentrations on oxygen uptake of minced Crocker 180 in the presence of malonate.*

Substrates	Initial Q_{O_2}	% of initial Q_{O_2}
---	7.0	74
4.5 mg. fumarate	6.8	86
2 mg. malonate	7.3	51
2 mg. malonate + 1.5 mg. fumarate	6.3	66
2 mg. malonate + 4.5 mg. fumarate	6.4	78
2 mg. malonate + 15 mg. fumarate	6.7	86

centrations were necessary to restore the respiration in the presence of 2 mg. malonate. The effect of adding C_3 monobasic acids corresponding to succinic, fumaric and malic, *i.e.* propionic, acrylic and lactic acids, was also investigated. Table IV shows that the monobasic acids produced no effects comparable with those of succinic and fumaric acids.

Table IV. *Comparison of the effects of propionate, acrylate and lactate with those of succinate, fumarate and malate on the oxygen uptake of minced JRS.*

Substrates	Initial Q_{O_2}	% of initial Q_{O_2}	Substrates	Initial Q_{O_2}	% of initial Q_{O_2}
---	8.5	69	---	8.5	69
Propionate 1.8 mg.	8.8	66	Succinate 4 mg.	8.2	95
Acrylate 1.8 mg.	8.5	67	Fumarate 4 mg.	8.3	84
Lactate 2.4 mg.	7.9	66	Malate 4.4 mg.	8.5	61

Annau.[1935] found that minced liver or kidney tissue produced acetone bodies in the presence of malonate. Similar accumulation of acetone bodies in the presence of malonate has now been shown (Table V) with the minced tumour slices (JRS or Crocker) in phosphate containing glucose. The nitroprusside

Table V. *Nitroprusside reactions.*

Substrates	JRS		Crocker 180
	Acetone bodies	Pyruvate	Acetone bodies
—	—	+	—
Malonate 2 mg.	+	—	+
Fumarate 4 mg.	—	+	—
Fumarate 4 mg. + malonate 2 mg.	—	+	—
Succinate + malonate	+	—	+

tests were performed after 2 hours' incubation with 0.25 g. tumour. Where acetone bodies were not formed by JRS tissue, pyruvate (as indicated by a blue coloration) was detected.

DISCUSSION.

This investigation shows that respiration of isolated tumour slices may be increased by addition of succinate or fumarate. The usual method of preparing tissue slices [Warburg, 1930] not only produces mechanical injury but also facilitates diffusion from the cells of essential substances such as oxygen carriers, which may thus become diluted beyond their effective or optimum concentration. The smaller the pieces of tissue, the more likely this is. Yet if the tissue slices are not sufficiently thin it is impossible for them to have enough oxygen for their needs when suspended *in vitro*.

Druckrey [1935] has suggested that injury is responsible for aerobic glycolysis of tumour slices. It seems important to point out that loss of oxygen carriers by diffusion would, by reducing the effective respiration, tend to increase aerobic glycolysis.

SUMMARY.

Respiration of tumour tissue (JRS and Crocker 180) is increased by addition of succinate or fumarate and decreased by addition of malonate. In this respect tumour resembles muscle, kidney and liver. The possibility that loss of readily diffusible oxygen carriers might affect the respiration of tumour slices is discussed.

We are indebted to the Imperial Cancer Research Fund for supplying us with strains of tumours. One of us (M.E.B.) has pleasure in thanking the House Committee of the Cancer Hospital for a scholarship held during the progress of this work.

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XXXVI. THE HYDROGEN ION DISSOCIATION CURVE OF THE CRYSTALLINE ALBUMIN OF THE HEN'S EGG.

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THIS paper is a report upon a somewhat extensive investigation of the hydrogen electrode titration of crystalline egg albumin. The work was undertaken with the object of examining the limitations of the method as a means of establishing a quantitative definition of the amphoteric properties of a protein. Crystalline egg albumin was chosen because it is probably the best accredited example of a molecularly homogeneous protein. The extensive work of Sørensen and of his colleagues, of Svedberg and of others indicates that of the common protein preparations egg albumin exhibits the greatest degree of constancy in composition, molecular weight and solubility under varying conditions of preparation and treatment. This protein has the further advantage that it is soluble in water over the whole titratable range of p_H .

The data which will be considered derive from observations gathered over a period of five years upon five distinct preparations of the protein. These preparations had been submitted to considerable variations in treatment prior to titration. Each was crystallised four times—one by the original method of Sørensen [1917], the others by a modification of this method in which sodium sulphate replaced ammonium sulphate as the salting-out agent. Two of the latter preparations were employed in the electrometric work without having been reduced to the dry state. Two were converted into a dry powder and stored for some time before use. One of the dry preparations was dissolved in water and titrated without further purification, the other after one more recrystallisation. Some details of the method of preparing crystalline egg albumin in the dry state with the aid of sodium sulphate will be found in the experimental section.

Each product was prepared for titration as a stock solution which had been dialysed in distilled water until the sulphate ion could not be detected in a dialysate after sixteen hours' contact with the solution. Varying amounts of dilute HCl or of NaOH were added to equal volumes of the stock solution and the mixtures were then diluted to a predetermined concentration of protein. The majority of the observations relate to systems containing 22–25 g. of protein in 1000 g. of water. In a few cases this value was reduced to 15 g. and in a few others raised to 45 g. The p_H of each mixture was determined in a rocking hydrogen electrode [Clark, 1928] at 25°, using a saturated calomel half-cell and a saturated potassium chloride liquid junction. The calomel half-cell was calibrated with the aid of 0.1 *N* HCl, for which we assumed a p_H of 1.075 [Scatchard, 1925]. The interval between the preparation of a mixture and the determination of its p_H did not exceed 1 hour.

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Calculations.

In the calculation of the hydrogen ion combined with the protein we made the following assumptions:

1. The albumin reacted to the addition of acid by reversible combination with hydrogen ions and to addition of base by reversible dissociation of hydrogen ions.

2. HCl and NaOH were completely ionised in the range of concentrations encountered. The activity coefficient of the hydrogen ion in any mixture containing HCl was the same as in a pure solution of HCl of the same concentration of chloride ions. The activity coefficient of the hydroxyl ion in any mixture containing NaOH was the same as the mean activity coefficient of NaOH in a pure solution of NaOH of the same concentration of sodium ions.

3. No diffusion potential existed at the liquid junctions.

The two latter assumptions are undoubtedly incorrect. The limitations which they impose upon the validity of the results will be discussed in a later section. The first assumption is the hypothesis whose utility in the analysis of titration data is under examination.

Symbols.

[Cl⁻] and [Na⁺] The respective molal concentrations of added acid or base in a reaction mixture.

[H⁺] and [OH⁻] The respective molal concentrations of hydrogen or of hydroxyl ions in the reaction mixtures at equilibrium.

g The mass of protein in grams in that volume of the mixture which contained 1000 g. of water.

h, H The equiv. of hydrogen ion combined with 1 g. and with 1 g.mol. (34,500 g.) respectively of the protein.

h₀ The equiv. of hydrogen ion combined with 1 g. of the protein in a mixture containing no added acid or base.

It was assumed that the isoelectric point was $p_{\text{H}} 4.90$ [Sørensen, 1925-28] and that at this p_{H} , $h=0$. A negative value of h indicates the dissociation of that amount of H⁺ from isoelectric protein. The introduction of h_0 is necessitated by the fact that the stock solutions were each somewhat removed from the isoelectric condition. That is to say they possessed individual initial values of h . It is these which we have represented by h_0 .

For electrical neutrality in any mixture

$$[\text{Na}^+] + [\text{H}^+] + g(h - h_0) = [\text{Cl}^-] + [\text{OH}^-] \quad \dots(1).$$

The values of [Na⁺] and of [Cl⁻] were calculated from the measured additions of acid and of base. This procedure involves the assumption that the protein does not combine with significant quantities of either of these ions. The values of [H⁺] and of [OH⁻] were obtained from the observed values of p_{H} with the aid of the relations:

$$p_{\text{H}} = -\log [\text{H}^+] - \log \gamma_{\text{H}^+} = \log [\text{OH}^-] + \log \gamma_{\text{OH}^-} - \log k_w \quad \dots(2),$$

where $k_w = 1.005 \times 10^{-14}$ [Lewis *et al.*, 1917]. Scatchard [1925] has made observations of the mean activity coefficient of HCl in solutions of this acid and from them has calculated a series of estimated values for γ_{H^+} . Our values for the latter have been taken from a curve drawn from Scatchard's results. We are not aware of any evaluation of γ_{OH^-} . We, therefore, assumed that, in the range of concentration with which we were concerned, it was substantially equal to $\sqrt{\gamma_{\text{Na}} \gamma_{\text{OH}^-}}$ in

corresponding solutions of NaOH. For the latter we used a curve drawn from the results of Harned [1925].

Equation (1) was solved for $h-h_0$. This was then plotted as a function of p_H , a separate curve being drawn for each stock solution. The value of $h-h_0$ on each curve at p_H 4.90 was determined from the curve. This, by definition, was the value of h_0 in the corresponding stock solution. Finally, all observations were brought together by plotting h as a function of p_H (Fig. 1). The figure includes

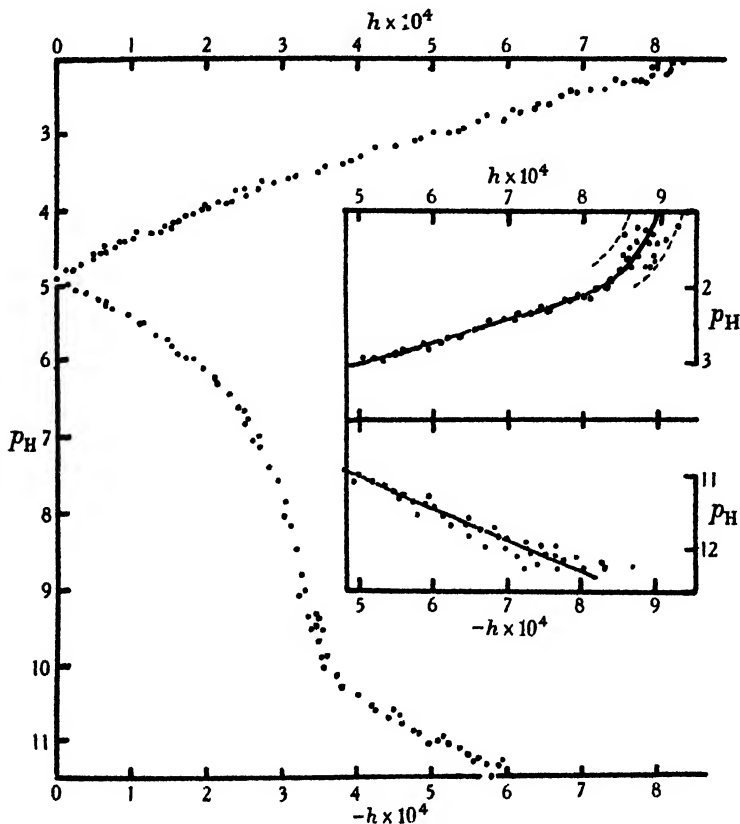


Fig. 1. H^+ ion combination curve of egg albumin. h —equiv. H^+ per g. protein.

about 180 points—observations on as many independent reaction mixtures. The only observations made in the course of this work which are not shown are the very few which through defects in the electrode system failed to give stable potentials. We have not attempted to indicate either the particular preparation or the particular concentration of protein to which each point refers. A careful inspection failed to reveal any distinct correlation between these variables and the deviations of individual points from the mean. Space does not permit the reproduction of even a representative selection of the data. We have therefore chosen a graphic analysis of the results. On a large reproduction of Fig. 1 a free hand curve was drawn through the assembled points and the ordinates of this curve were measured at small increments of p_H . These are recorded in Table 1. Within the p_H range 2–11.5 no observations deviate from this mean curve by

Table I. *Hydrogen ion combining capacity of egg albumin.*

p_H	Equiv.		p_H	Equiv.	
	$\times 10^5$ per g.	Per g.mol.		$\times 10^5$ per g.	Per g.mol.
1.5	89.0	30.7	6.50	- 23.0	- 7.9
1.75	86.0	29.7	6.75	- 24.8	- 8.5
2.00	81.2	28.0	7.00	- 26.2	- 9.0
2.25	74.5	25.7	7.25	- 27.4	- 9.5
2.50	66.4	22.8	7.50	- 28.5	- 9.8
2.75	58.5	20.2	7.75	- 29.8	- 10.3
3.00	49.2	17.0	8.00	- 30.8	- 10.6
3.25	40.6	14.0	8.50	- 32.0	- 11.0
3.50	32.2	11.0	9.00	- 32.8	- 11.3
3.75	25.0	8.6	9.50	- 34.5	- 11.9
4.00	19.0	6.6	10.00	- 37.0	- 12.8
4.25	13.0	4.5	10.25	- 39.4	- 13.6
4.50	7.0	2.4	10.50	- 42.5	- 14.7
4.75	2.0	0.7	10.75	- 46.0	- 15.9
5.00	- 2.2	- 0.8	11.00	- 50.4	- 17.4
5.25	- 6.0	- 2.1	11.25	- 55.7	- 19.2
5.50	- 10.4	- 3.6	11.50	- 61.0	- 21.0
5.75	- 14.0	- 4.8	11.75	- 67.0	- 23.0
6.00	- 17.8	- 6.2	12.00	- 74.0	- 25.7
6.25	- 20.5	- 7.1			

more than ± 0.5 equiv. per g.mol. of protein. The loss of precision which appears outside these limits will be considered below. In the meantime we submit that Table I defines a curve, which within the range p_H 2–11.5 represents a physical property of the protein reproducible within an error which is certainly less than one equiv. per g.mol. of protein. One qualification must be entered. The curve reported is valid only for systems from which electrolytes other than the reactants have been excluded. In a later communication we hope to report on the rather profound effects of changes in ionic strength due to addition of neutral salts.

A number of electrode titrations of egg albumin are recorded in the literature. Unfortunately the majority of these are reported in the form of small scale curves with which precise comparison is impossible. In many cases moreover the curve is modified by the presence in the protein preparation of significant concentrations of ammonium sulphate. Cohn [1925] collected the results available at that time and showed that they were in general conformity with one another and with unpublished observations of his own. Of the results quoted by Cohn we have taken the curves of Loeb [1920] and of Hitchcock [1920]. After readjustment of the isoelectric point we find that the results of Hitchcock which cover the range p_H 2–5 are in satisfactory agreement with Table I. Loeb's results extend from p_H 2 to 11. From the acid extreme to p_H 8 they do not deviate from our curve by much more than 0.5 equivalent per g. mol. The alkaline end of the curve however departs seriously from ours, a discrepancy which may in part be attributed to the presence of ammonia. Recently Prideaux and Woods [1932] have published an extensive series of observations which should have proved a valuable basis of comparison. Unfortunately they lack consistency with each other to a degree which is entirely contrary to our experience and which forbids any attempt at analysis.

The submission of a standard hydrogen ion dissociation curve for a protein requires proof that the observations relate to strictly reversible equilibria. We have repeatedly demonstrated to our satisfaction that the electrode potentials of reaction mixtures rapidly attained stable values. These remained constant (within a few tenths of a millivolt) for periods up to 48 hours, provided that the mixture was within the p_H range 2.5–11.0. We have found moreover that within

these limits a system may be moved up and down the curve in a strictly reversible manner. In making such tests it is important that the electrolyte concentration be maintained substantially constant. Precautions must also be taken to insure reproducibility of liquid junctions and to prevent diffusion of potassium chloride from them into the reaction mixtures during the periods of observation.

Below p_H 2.0 and above p_H 11.5 it is possible to demonstrate slow but consistent drifts in potential with time. In acid solutions these are in the direction of mounting values for p_H . In alkaline solution they proceed in the opposite direction. The changes are irreversible and must be assumed to be due to irreversible changes in the hydrogen ion dissociating capacity of the protein. Now the first recognised change which takes place in egg albumin under the influence of acid or of alkali is the formation of metaproteins. We are at present engaged upon a study of these products and find that separated acid metaprotein does dissociate considerably more H^+ and separated alkali metaprotein does combine with considerably more H^+ than the native albumin. Preliminary measurements have been made of the magnitude of these irreversible changes and of their rates as a function of p_H . The results, which will be published shortly, are such as to indicate that titrations may not be conducted beyond the p_H limits of 2 and 11.5 without danger of distortion from these irreversible processes.

Earlier in this paper it was acknowledged that the validity of the curve which we are reporting is limited by the errors involved in the assumption respecting (a) diffusion potentials and (b) the activity coefficients in equation (2). The presence of a significant diffusion potential will be reflected in an error in the observed p_H and therefore in the calculated value of $[H^+]$. An error in the assumed value of either γ_{H^+} or γ_{OH^-} will, likewise, introduce an error into $[H^+]$. Now in acid solution g, h is substantially equal to $[Cl^-] - [H^+]$ and in alkaline solution to $\frac{k_w}{[H^+]} - [Na^+]$. It follows that the sources of error under consideration will affect h increasingly the more closely $[H^+]$ approximates to $[Cl^-]$ or $[OH^-]$ to $[Na^+]$, *i.e.* the higher the ratio of free HCl to total chloride or of free NaOH to total sodium. This consideration, be it said, was the one which led us to choose for the activity coefficients the values obtaining in pure solutions of acid or base. The choice becomes the more nearly correct the more seriously an error in it would affect the results.

In order to determine the practical limits of p_H imposed on the method of titration by these sources of error, we will assume the possibility of a diffusion potential of about 1 mv. and an error in the assumed activity coefficients of 5%. A simple calculation will show that if any one of these were operative alone it would lead to an error of about 5% in $[H^+]$. Taken together a possible error of 10% arises. Now consider a 2.5% solution of albumin. With the aid of Table I it may be calculated that an error of 10% in $[H^+]$ would introduce an error of about 4 equiv. per g.mol. at p_H 1.5 or 12.5 and of about 1 equiv. at p_H 2.0 or 12.0. If a precision of at least 1 equiv. per g.mol. is to be attained, it would appear that we may not safely continue the titration curve beyond the limits p_H 2–12.0. Increase in the concentration of protein may permit a slight, but only slight, extension of these limits. These considerations together with those respecting irreversible changes in the protein lead to the choice of p_H 2–11.5 as the limits within which electrometric determinations of the hydrogen ion dissociation curve are dependable.

Maximum hydrogen ion combining and dissociating capacities.

Inspection of Fig. 1 will lead to the conclusion that within the acceptable range p_H 2–11.5 there is no evidence of the attainment of either a maximum or a minimum value of h . Below p_H 2 however the curve does turn definitely in a direction which, with some plausibility, we may extrapolate to a maximum value of about 0.9 milliequiv. per g. This is indicated in the upper inset curve of Fig. 1. The continuous curve is drawn for a maximum combining capacity of 31 equiv. of H^+ per g.mol. (0.89 milliequiv. per g.). The two broken lines are the corresponding curves for 30 and for 32 equiv. per g.mol. respectively. Although theoretically this extrapolation is subject to large errors, we are inclined to accept as the maximum hydrogen ion combining capacity of egg albumin the value of 31 ± 1 equiv. per g.mol. (0.87–0.93 milliequiv. per g.).

At the alkaline extremity of the curve on the other hand there is no indication of a maximum dissociating capacity even beyond p_H 12. There are evidently present in the protein groups which dissociate hydrogen ions only in strongly alkaline media. Although there is no indication of a stoichiometric end-point at the alkaline extremity of the curve, a distinct break in the curve does appear between p_H 8 and 9. This corresponds with the dissociation of 11 equiv. of hydrogen ion per g.mol. It occurs in so readily controllable a range of p_H that it should prove a very useful point about which to orient measurements of the amphoteric behaviour of the protein.

In forthcoming papers we hope to report on the changes in the curve which accompany (a) changes in the electrolyte concentration and (b) the presence of formaldehyde.

EXPERIMENTAL.*The preparation of crystalline egg albumin with the aid of sodium sulphate.*

The following method was found convenient. The preparations used in this work have been of the order of 100–400 g. each.

A solution of Na_2SO_4 was prepared by dissolving 400 g. of the anhydrous salt in 1 litre of warm water. This solution which contains 36.7 g. of salt in 100 ml. must be kept at a temperature above 30° to prevent crystallisation. Having collected the whites of fresh eggs and broken up the membranes, the volume was measured and an equal volume of the salt solution added. The mixture was stirred for some time and then after 1 to 2 hours the precipitate was removed by filtration or in the centrifuge. A solution of 0.2 N H_2SO_4 was slowly added to the filtrate whilst the latter was stirred mechanically. Titration was continued until the p_H was about 4.6–4.8. A test on a few drops of the mixture with bromocresol green proved sufficiently accurate. It was unusual for any permanent precipitate of protein to separate during the titration but, if it did do so, it was redissolved by cautious addition of water. After the desired p_H had been attained, stirring was continued and anhydrous Na_2SO_4 was added slowly until a permanent opalescence developed. When crystallisation of the protein became clearly evident the mixture was decanted from any solid Na_2SO_4 which remained undissolved and placed on one side for a day or two. The whole of the process must be conducted in a room not cooler than 20° or it will be impossible to achieve a high enough concentration of salt to effect crystallisation of the protein.

The crystalline material was removed by filtration or in the centrifuge and redissolved in a volume of water about equal to the original volume of egg white. Recrystallisation was then effected by addition of solid Na_2SO_4 accompanied by stirring. From 140 to 180 g. of the anhydrous salt was required for each litre of

solution. After two further recrystallisations the final product was either brought into solution and dialysed or converted into a dry powder. In the former case it was possible by extracting the crystalline mass with ice-cold water to obtain a concentrated solution of protein which contained only about 5% of sodium sulphate.

By spreading the mass of crystals and mother-liquor thinly in front of a fan in a warm room it was rapidly reduced to a dry powder. Little denaturation occurred during desiccation. The product was readily ground to a fine powder which sampled reproducibly and in which the protein had retained its ability to crystallise after storage at room temperature for 2 years. The compositions of two such preparations have been

	%	%
Soluble protein	63	70
Insoluble protein	3	1.8
Inorganic	28	23
Water	6	5.2
	100	100

After the first preparation had been stored for 2 years a sample was recrystallised. 93% of the soluble protein (determined two years earlier) was recovered in the normal crystalline form.

The main advantage to us in the use of Na_2SO_4 for crystallisation was the elimination from our products of ammonia. Dialysed solutions of egg albumin which have been crystallised from $(\text{NH}_4)_2\text{SO}_4$ retain amounts of NH_4^+ which depend on the p_{H} of the solution. If specific action is not taken to remove this the observed titration curve of the protein will include the titration of NH_4^+ in the region of p_{H} 9–10. Now egg albumin is often crystallised at a p_{H} above 5.0. A simple calculation from Table I will show that the ammonia retained by such a protein will at p_{H} 10 dissociate an amount of hydrogen ion equal to 10% of that dissociated by the protein itself. Had the initial p_{H} of the protein been 5.25 the error introduced would have been 20%.

Analytical.

The concentrations of the various dialysed stock solutions were of the order of 7–10%. A 5 ml. pipette was calibrated for the weight of the solution which it delivered under standard conditions. The dry weight, ash, total nitrogen and ammonia (none was found) in this volume of solution were determined. The dry weight was determined at 105°, the ash as sulphate and the ammonia by distillation *in vacuo* with $\text{Mg}(\text{OH})_2$. In common with other observers we have had trouble with the determination of nitrogen in egg albumin by the Kjeldahl method. Various modifications of this method give different results and some methods are inconsistent in their behaviour. On the whole the Arnold-Gunning modification has proved the most satisfactory. In the calculations involved in the titrations we have preferred to use the ash-free dry weight rather than the protein calculated from the total nitrogen.

Electrode titrations.

The aliquots of the stock solutions for the various reaction mixtures were measured with the calibrated 5 ml. pipette. The first observation of the potential was made after 10 min. equilibration in the electrode vessel. The second reading was made after a further period of 5 min. in a fresh atmosphere of hydrogen. If these did not agree within 0.2–0.3 mv. (in the substantially unbuffered range of

p_H 8–9 a somewhat larger error was accepted) a third reading was taken. If a pair of these readings did not agree the sample was rejected and the electrode discarded. Each reading, of course, corresponded with a fresh liquid junction. The electrodes employed were thinly palladised or platinised platinum.

SUMMARY.

1. On the basis of an extensive series of observations of the hydrogen electrode potentials of the systems egg albumin-HCl and egg albumin-NaOH in dilute aqueous solution, data are submitted for the construction of a hydrogen ion dissociation curve of egg albumin.

2. An error not greater than ± 0.5 equiv. of hydrogen per g.mol. of albumin (mol. wt. 34,500) is claimed within the range of p_H 2–11.5.

3. Certain facts which limit the precision of the titration method are discussed.

4. The hydrogen ion combining capacity of egg albumin probably reaches a maximum of 30–32 equiv. per g.mol. slightly below p_H 2. At the alkaline extreme of p_H 11.5–12.0 there is no evidence of a stoichiometric end-point indicative of a maximum dissociating capacity. Such an end-point is however observed between p_H 8 and 9 and corresponds to the dissociation of 11 equiv. of hydrogen ion per mol.

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XXXVII. THE EFFECT OF FORMALDEHYDE ON THE HYDROGEN ION DISSOCIATION CURVE OF EGG ALBUMIN.

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It is well known that the cations of many nitrogenous bases which cannot be directly titrated with alkali in aqueous solution submit to titration in the presence of a suitable concentration of formaldehyde. This is true of certain of the cationic groups in the proteins and their hydrolytic products. In the case of the natural amino-acids and their peptides, the various cationic groups which may occur are (a) primary amino-groups, (b) the imino-groups of proline and hydroxyproline, (c) the guanidino-group of arginine and (d) the glyoxaline group of histidine. Only the first two types however react with formaldehyde to an extent sufficient to produce any profound change in their acidic properties. Birch and Harris [1930] first employed the hydrogen electrode to investigate the nature of these changes. More recently, Levy [1933; 1935] has used the same method in an extensive study of the principles involved in the "formol" titration. It appears that the acid-base changes accompanying the addition of formaldehyde may be quantitatively described by an increase in the apparent hydrogen ion dissociation constant of certain cationic groups. Levy has shown that the constants of the cations of primary NH_2 groups vary with the square of the concentration of formaldehyde above certain low limits of concentration. In the cases of proline and hydroxyproline on the other hand the change in the apparent dissociation constant is directly proportional to the formaldehyde concentration.

The present paper is a report on an extension to egg albumin of the electro-metric method of conducting formaldehyde titrations. The electrode behaviour of egg albumin solutions is decidedly less satisfactory in the presence of formaldehyde than in its absence. Nevertheless reproducible titration curves may be traced if occasional irrational observations and the electrodes responsible for them be rejected. The results meet the purposes of the present inquiry, though some features of the reaction remain obscure. It appears that the addition of formaldehyde is accompanied by an immediate readjustment of the acid-base equilibrium. This is followed however by slow secondary changes which persist for many hours and are accompanied by small but persistent changes in hydrogen electrode potential. In a period of one hour however these changes in potential are small in comparison with the large initial change. We have therefore ignored them and have accepted the substantially constant potentials observed during the first hour after the addition of formaldehyde. In doing so we believe that our curves faithfully reproduce the gross acid-base changes which accompany the usual process of formaldehyde titration.

The method of calculation differs from that used in the first paper of this series [Kekwick and Cannan, 1936] only to the extent that the anion of form-

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aldehyde contributes to the balance of ions which establishes electrical neutrality in the system. Equation 1 of that paper becomes

$$[H^+] + [Na^+] + g(h - h_0) = [Cl^-] + [OH^-] + [F^-] \quad \dots (1A),$$

where $[F^-]$ is the molal concentration of the anion of formaldehyde. In order to calculate this from the total concentration of formaldehyde ($[F]$) we have used the relation given by Levy [1934]

$$p_H = 12.87 + \log \left[\frac{[F]}{[F^-]} - 1 \right] - 0.19 [F] \quad \dots (2).$$

The final term in this equation is an empirical correction introduced by Levy to accommodate the effect of the concentration of formaldehyde upon its apparent p_K . The correction is of such magnitude as to imply changes in the activities of the reactants which are much greater than is to be expected from the changing composition of the system. Presumably this dependence of p_K upon $[F]$ results from the presence of secondary equilibria which are dependent upon the primary dissociation of a hydrogen ion. However this may be, Levy's equation permits the calculation of $[F^-]$ in equation (1A) though its approximate character introduces a new source of error into the calculated values of h . This error will become serious at p_H values at which $[F^-]$ attains values comparable with those of g , h and thus places a new upper limit to the p_H to which titrations may be carried with precision. When $[F] = 1$ this limit will be close to p_H 10 and when $[F] = 0.1$ to p_H 11.

In Fig. 1 the hydrogen ion dissociation curve of 1 g.mol. of egg albumin [Kekwick and Cannan, 1936] in dilute aqueous solution is compared with the corresponding curves which were observed in the presence of four concentrations

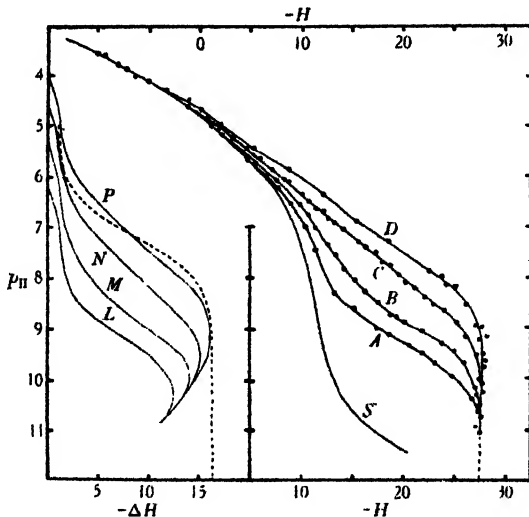


Fig. 1. H^+ dissociation curves in the presence of formaldehyde. Curves *S*, *A*, *B*, *C* and *D* correspond to 0, 0.1, 0.2, 0.6 and 1.26 *M* formaldehyde respectively. Curves *L*, *M*, *N* and *P* are the comparison curves (see text) for 0.1, 0.2, 0.6 and 1.26 *M* formaldehyde respectively. H = equiv. H^+ per g.mol. protein.

of formaldehyde (0.1, 0.2, 0.6 and 1.26 *M*). At a sufficiently low p_H all the curves coincide. As the p_H rises the curves successively diverge from the control, become roughly parallel and finally converge again to a common limiting value of $-H = 27$ –28 equiv. per g.mol. A graphic analysis of these curves is illustrated

by the inset curves on the left of Fig. 1. At successive small increments of p_H we determined the differences in H, (ΔH), between the value in water and in each concentration of formaldehyde. ΔH was then plotted against p_H to give the family of comparison curves shown. Discussion will be facilitated by designating as the F_x groups those groups in the protein whose affinity for H^+ is notably decreased by formaldehyde. The subscript "x" will designate the particular concentration of formaldehyde under discussion. As a first approximation, we may regard these curves as the resultant of the H^+ dissociation curves of the F_x groups and the H^+ association curve of the F_0 groups. The latter will be represented by the common reentrant section above p_H 10 and the former by the sections from p_H 4 to 10. These, it will be seen, vary in position with the concentration of formaldehyde. The four curves attain different maximum values of H indicating an overlapping of the F_0 and F_x curves. Curve *P* for 1.26 *M* however exhibits a fairly flat plateau for $-\Delta H = 16-17$. We may conclude that at this and higher concentrations of formaldehyde there is little overlapping and the maximum value for $-\Delta H$ corresponds to the stoichiometric number of F groups in the protein. We are inclined to place the latter at a value of 17-18 per g.mol. of egg albumin.

Consider now only the section of each curve which extends from p_H 4 to 10. Each curve exhibits a small segment between p_H 4 and 6 amounting to 1-2 equiv. per g.mol. Since the calculated values of ΔH are probably subject to an error of ± 0.5 equiv. per g.mol. one may not place great emphasis on this small segment. It appears, however, in all the curves and its position on the p_H scale varies in an orderly fashion with $[F]$. We may conclude that 1-2 of the F groups are distinct in type from the remainder.

Confining consideration to the latter the comparison curves suggest that they are uniform in type. We have attempted to indicate this by superimposing on the appropriate section of the $F_{1.26}$ comparison curve a theoretical dissociation curve (broken line) for 16 groups. The comparison curve is manifestly symmetrical. Its departure from the theoretical curve is of the type that might be expected as a result of the spread of p_K values in a polyvalent substance with uniform groups.

In terms of the polypeptide theory of protein structure the F groups to be expected are the $\epsilon-NH_2$ groups of lysine together with a single $\alpha-NH_2$ group at the end of the polypeptide chain. It would be tempting to identify the 1-2 unique F groups indicated above with this $\alpha-NH_2$ group. There would then remain 16-17 lysine groups. This will be a minimum value for the following reason. The p_K values which have been found for $\alpha-NH_2$ groups in simple polypeptides are close to 8. The low p_H at which our F group appears in the presence of formaldehyde is also consistent with a low p_K in its absence. If the latter were at any value below 9 then the total number of F groups betrayed by the maximum on the comparison curve would not include this one unique group because it would have been dissociated completely in the absence of formaldehyde at the p_H of the maximum value of $-\Delta H$. In such case $-\Delta H$ measures only the lysine groups. Until we can establish the position which the unique group occupies in the curve for aqueous solution we must be content to conclude that titration with formaldehyde indicates the presence of 16-18 lysine groups. In so far as the identification of the unique group with $\alpha-NH_2$ is plausible the higher value for lysine is the more probable.

The recent careful analyses of egg albumin by Vickery and Shore [1932] and by Calvery [1932] agree in finding not more than 12 mols. of lysine in 1 mol. of albumin. The formaldehyde titration suggests 16-18. We sought to clarify this

discrepancy by a determination of the number of groups in egg albumin which react with nitrous acid. Choosing conditions of reaction under which the terminal NH_2 group of lysine itself reacts quantitatively and making allowances for secondary reactions, we find that 19–20 groups react with nitrous acid (Fig. 2). One of these will be the $\alpha\text{-NH}_2$ group leaving 18–19 presumed lysine groups. The agreement seems satisfactory when the higher value for the formaldehyde titration is accepted. In any case the important point is that the nitrous acid method

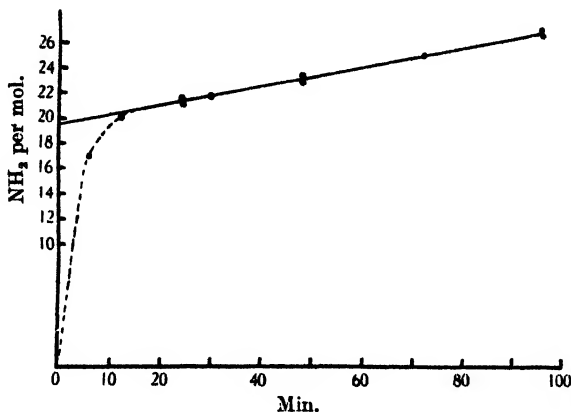


Fig. 2. The NH_2 groups of egg albumin from the nitrous acid reaction showing the effect of reaction time at 21–23° on the results.

and the formaldehyde titration both indicate the presence of an amount of free NH_2 groups much greater than the amount of lysine which has actually been isolated from the hydrolysed protein. The discrepancy is confirmed by determinations of the nitrogen distribution of the products of hydrolysis of egg albumin. Using the method of Van Slyke, we find that the nitrogen of the lysine fraction corresponds to 17–18 mols. of this amino-acid in a molecule of protein. It is doubtful however if much weight can be put on the results of this method of determining lysine.

It is of some interest to attempt an estimate of the relation between $[F]$ and the p_K values of the F groups. This may be done in a rather empirical manner from the comparison curves by reading off at a given ΔH the p_H values corresponding to the several concentrations of formaldehyde. The following results may be quoted to illustrate the conclusion that $\frac{[\text{H}^+]}{[F]^2}$ is substantially constant when F is greater than 0.2.

$-\Delta H$	$[F]$	p_H	$p_H + 2 \log [F]$
7.5	0.10	9.13	7.13
7.5	0.20	8.67	7.27
7.5	0.60	7.65	7.30
7.5	1.26	7.10	7.30

This conclusion which presumably defines the variation of the p_K of the lysine groups with $[F]$ conforms to the behaviour of NH_2 groups in general as established by Levy.

There remains for consideration the section of the comparison curves above p_H 10. This is to be interpreted as the hydrogen ion association curve of the lysine groups in the absence of formaldehyde. Were it possible to trace the full course of

this curve we should have a measure of the effective p_K of the lysine groups. Unfortunately the errors of the formaldehyde titration at high p_H do not permit this. The available segment of the curve is however consistent with the conclusion that the dissociation of the lysine groups of the protein in aqueous solution simulate those of an acid of p_K close to 11.

In the light of the data which have been presented we may in conclusion submit certain recommendations respecting the use and interpretation of formaldehyde titrations of protein solutions conducted with the aid of indicators. If it is desired to measure the total F groups other than $\alpha\text{-NH}_2$, the most satisfactory conditions would appear to be (a) adjustment of the solution to an initial p_H 9, (b) addition of neutral formaldehyde to a concentration of 2 M and (c) titration of the mixture back to p_H 9. At this p_H the buffering due to the protein alone and to the formaldehyde alone is small and the titration of the F groups is substantially complete. The end-point is fairly sharp. Should steps (b) and (c) lead to an important change of total volume then a correction must be introduced for the H^+ dissociated by the formaldehyde in this increased volume. It is probable though not established that the same conditions should prove the best for the majority of proteins.

If the titration is to include $\alpha\text{-NH}_2$ groups, then the initial adjustment of p_H should be to p_H 6.5. Owing to the rather effective buffering power of the protein in this region, this adjustment will not be very precise. The formaldehyde is then added and the titration conducted to p_H 9. Such a titration includes the H^+ dissociated by the protein between p_H 6.5 and 9 in the absence of formaldehyde. This can be ignored if relative changes in $\alpha\text{-NH}_2$ groups are sought but may be approximately controlled by an independent titration in the absence of formaldehyde. It should be stated in conclusion that salt effects on the ionisation of the protein are so large that serial titrations conducted without control of the ionic strength may be subject to serious errors.

EXPERIMENTAL.

Electrometric titrations.

The technique of titration was the same as that reported in the first paper of the series [Kekwick and Cannan, 1936], except for the addition to each reaction mixture of a measured volume of a standard formaldehyde solution. The latter was prepared and standardised after the manner described by Levy [1933]. The titration curve for 0.6 M formaldehyde shown in Fig. 1 was calculated from the results of titrations of two different protein preparations—both of which had been used for titrations described in the first paper. One of these was a Na_2SO_4 preparation which had not been dried. The other was one of the dried preparations. The titration curve for 0.1 M formaldehyde refers to the former preparation only and the curves for 0.2 and 1.26 M formaldehyde to the latter only.

The nitrous acid reaction.

The amount of nitrogen formed when nitrous acid reacts with egg albumin under the conditions of the Van Slyke manometric method was found to vary with the time of reaction. In Fig. 2 are shown the results obtained when the time was varied from 6 to 96 min. at 21–23°. The results are calculated as equiv. of NH_2 per mol. of egg albumin. It is clear that the rapid initial reaction is followed by a relatively slow increase which is approximately linear in rate. We may presume that the former is the reaction which is due to the presence of

NH₂ groups in the native protein. We believe therefore that the most reliable determination of these is secured by extrapolating to zero time the linear rate of secondary change.

The nitrogen in the basic fraction of hydrolysed egg albumin.

Van Slyke's method [1911] was followed with one modification. The original precipitate of the phosphotungstates was recrystallised once from a volume of washing fluid (2.5 % H₂SO₄ containing 0.5 % phosphotungstic acid) equal to the volume of the mixture in which it had been formed originally. It was then washed and treated in the manner described by Van Slyke. The recrystallisation was carried out with the hope of reducing the danger of including neutral amino-acids in the final lysine nitrogen.

About 8 g. of albumin were used in each of three determinations. The three experiments agreed within the limits arginine 10.5–11.0, histidine 2.8–3.1 and lysine 17.7–18.4 equiv. of amino-acid per mol. The figures for arginine and histidine agree well with those of Vickery and Shore, though it must be pointed out that our results for histidine are subject to a large error due to the solubility correction being a large fraction of the whole. Our figure for lysine is, as has been discussed, much larger than the values found by authors using isolation methods.

SUMMARY.

1. Hydrogen ion dissociation curves of crystalline egg albumin in 0.1, 0.2, 0.6 and 1.26 *M* formaldehyde are reported.
2. From an analysis of these curves it is concluded that the egg albumin molecule contains 16–18 NH₂ groups other than α -NH₂ groups. These are tentatively identified with the ϵ -NH₂ groups of lysine.
3. The reaction of egg albumin with nitrous acid indicates the presence of 19–20 NH₂ groups per g.mol. The lysine-nitrogen of the phosphotungstate fraction of the hydrolysed protein corresponds with 17–18 molecules of lysine per mol.

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XXXVIII. DETERMINATION OF CARBON AND NITROGEN BY THE ACTION OF CHROMIC ACID UNDER REDUCED PRESSURE.

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DURING the course of work on the anaerobic decomposition of plant materials in the soil, the need was felt for a rapid and accurate method for the determination of carbon in extracts and solid residues, which in certain cases, *e.g.* in the determination of lignin, could also estimate the amount of nitrogen present in the same sample. The chromic acid method suggested itself and was examined in some detail to ascertain its suitability for the purpose in view. This method has been used by numerous workers for such widely different materials as soils, industrial wastes, coals, iron, steel and various organic compounds.

The low results obtained for carbon by the earlier workers have been shown [Friedmann and Kendall, 1929] to be due to insufficient heating, low temperature, too much dilution *etc.*, and it is now generally admitted that with improved manipulation the method is capable of yielding results as accurate as those obtained by the dry combustion procedure.

Among the workers who have examined the suitability of the method for the simultaneous determination of nitrogen, Fritsch [1896], Kruger [1894], Anderson and Schutte [1924], Antipov-Karataiev and Fillipova [1932], Brown [1927] and Tiurin [1933] have reported satisfactory agreement between the Kjeldahl values and those obtained by alkaline distillation of the residue after chromic digestion, whilst others [Dafert, 1888; Robertson, 1916; Shewan, 1935; Subrahmanyan *et al.*, 1934] have found that the method yielded low results. The low values have been attributed to the presence of chlorine [Robertson, 1916; Anderson and Schutte, 1924], to the oxidising action of chromic acid, to the structure of the compound [Shewan, 1935] and to a portion of the nitrogen being retained by the chromium in the oxidising mixture [Narayanayya and Subrahmanyan, 1935]. The author has recently found that the recovery of ammonia by the method bears a definite relation to the structure of the compound and that the loss occurs by a portion of the nitrogen being oxidised to nitrate and the rest evolved in gaseous form, as nitrous oxide in the cases examined.

APPARATUS AND PROCEDURE.

The apparatus used in the present investigation, shown in Fig. 1, is a modification of that used by Adams [1934], though the manipulative details show much difference. The Kjeldahl flask *A* of about 500 ml. capacity is fitted in the neck with a boiling-tube *B* having its bottom punctured and packed with glass-wool to serve as a trap for the absorption of SO_2 fumes [Pollard and Forsee, 1935]. The inlet tube *C* connects with the annular space between *B* and *A* and carries a cock *F* by means of which the admission of air into the apparatus can be

regulated. The exit tube *D* is connected respectively to a test-tube *L* containing H_2SO_4 of constant boiling-point [White and Holben, 1925] for retaining SO_3 fumes, kept in a bath of cold water, a CaCl_2 drying tube *M*, two weighed tubes *P* and *N* packed with "sofnolite" and anhydrous CaCl_2 , as shown in the figure, for the absorption of CO_2 and finally to a CaCl_2 drying tube *Q* and suction pump *S*.

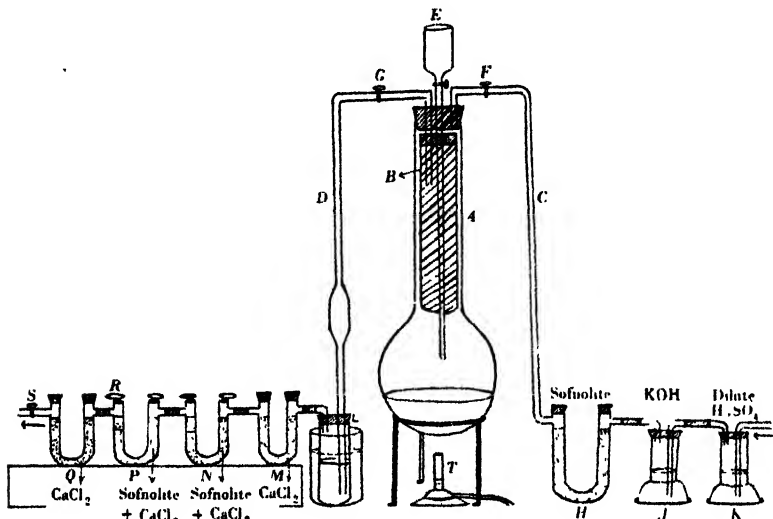


Fig. 1.

The manipulative details adopted were as follows. A weighed amount of the substance under investigation (to yield 0.2–0.4 g. of CO_2) is transferred into the Kjeldahl flask preferably through a glass tube which is subsequently washed with 5–10 ml. of distilled water. The flask is fitted into place, cock *F* is closed and the required amount of CrO_3 solution, at the rate of 1 ml. of aqueous 50% (wt/volume) CrO_3 solution per 100 mg. of CO_2 expected, is placed in the thistle funnel *E*. Suction is then started, most of the CrO_3 solution let in and the sides of the funnel washed with about 2 ml. of water, to prevent the formation of a precipitate on the subsequent addition of H_2SO_4 and possible jamming of the cock. Sufficient H_2SO_4 is then added, in small portions at a time, to make the ratio of H_2SO_4 : total water (including CrO_3 solution) equal to 2 : 1 by volume or slightly above. Higher ratios than 3 : 1 produce a cake at the bottom of the flask in the case of soils, which is hard to remove later by mechanical rubbing or warming with water. It can be removed, however, by warming with alkali followed by treatment with dil. HNO_3 .

After all the H_2SO_4 has been added, cock *F* is partially opened to admit a slow stream of air, freed from NH_3 and CO_2 , at the rate of 1 or 2 bubbles per second. The rate at which air is removed from the flask being much greater than that at which it is admitted, there is throughout the experiment low pressure maintained in the flask of the order of 3–4 in. of mercury. This low pressure, besides promoting vigorous boiling at a lower temperature and the avoidance of excessive fumes, markedly improves the efficiency of the absorption of the CO_2 evolved. The admission of air serves partly to carry off the CO_2 evolved and partly to cool the neck of the flask and condense fumes of SO_3 formed. In spite of this arrangement and the glass-wool trap *B*, a portion of the fumes was found always to be carried

forward into the exit tube *D*, but was effectively retained by the 98.3 % H_2SO_4 in tube *L*. It may be stated that used portions of the acid in *L* could be collected together, heated for several hours and used over again.

About 20–30 minutes' digestion of the contents of the flask at boiling-point was found to be sufficient for most soils and plant materials, but where derivatives of pyridine, quinoline, *iso*quinoline or the tetrammonium compounds such as betaine and choline are present, a longer time may be required depending on the nature of the compounds present. In these cases, completion of oxidation could be tested either by stopping the digestion at half-hour intervals and weighing the amount of CO_2 formed, or better without interrupting the oxidation, by having two sets of "sofnolite" tubes connected in parallel and collecting the CO_2 evolved in alternate sets per half-hour. Suitable blanks should be run in each case. At the end of the digestion, cocks *R* and *S* are closed and cock *F* is opened in stages so as to admit a regulated stream of air into the apparatus and equalise the pressure inside and outside, after which the "sofnolite" tubes *P* and *N* are detached and weighed.

Direct distillation with alkali, without pretreatment, of the digested residue gives only the amount of ammoniacal nitrogen present. It will be seen later that in the cases of soils and plant materials, for nitrogen values of 5 mg. and above, this value is uniformly lower than the Kjeldahl figure by 10 % and this correction could be applied. Where, however, the amount of nitrogen present is about 5 mg. or less, the difference between ammoniacal nitrogen formed and the total nitrogen found by the Kjeldahl method is due to a quantitative oxidation to nitrate which is held in solution by the H_2SO_4 present. The total nitrogen in such cases can be estimated as follows.

The excess of chromic acid is reduced by gentle warming and addition of solid sodium sulphite in small quantities at a time, testing for completion of reaction with starch-iodide solution used as an external indicator. A few pieces of solid paraffin and pumice stone are added and most of the H_2SO_4 neutralised by careful addition of NaOH in small quantities, keeping the flask cooled under the tap. 30–40 ml. of 50 % NaOH and 1–2 g. of Devarda's alloy are then added and the distillation conducted as usual. The addition of paraffin and removal of most of the acid by preliminary neutralisation avoids excessive frothing and a too vigorous reaction in the early stages. A trace of the paraffin usually passes over but this does not interfere with the clear definition of the end-point. Even this can be avoided by using paraffin which has been previously boiled for some time with alkali.

ESTIMATION OF CARBON.

Influence of other substances. The addition of metallic catalysts was found to have little effect on the course of the reaction. H_3PO_4 was found to moderate the vigour of the reaction and hence a mixture of H_3PO_4 and H_2SO_4 recommended by some workers [Schollenberger, 1916; Assoc. Off. Agric. Chem., 1930] was not used. Sulphur, sulphide and sulphites did not interfere.

Chlorides seriously interfered with the accuracy of the carbon determination on account of chlorine passing over; this could be conveniently avoided by adding 1 to 2 g. of HgO to the substance in the Kjeldahl flask before the addition of CrO_3 and H_2SO_4 [cf. Florentin, 1924; Subrahmanyam *et al.*, 1934] and by adding H_2SO_4 in small quantities with gentle rotation of the flask.

The following procedure was tried with success in cases where the addition of HgO is for other reasons undesirable. After the addition of CrO_3 solution, the suction is continued for 2 or 3 min. to ensure a sufficiently low pressure in the

flask, then cock *G* is closed and cock *F* opened slightly to allow entry of air at the rate of a bubble per second. H_2SO_4 is added, in small portions at a time, with gentle rotation of the flask. Only sufficient of the substance should be taken to yield 100–200 mg. of CO_2 , to avoid escape of CO_2 by bubbling upward through the thistle funnel *E*. At first a white cloud of HCl is formed, which however is kept down by the flow of air from the inlet tube *C*, and after a few seconds the bottom of the flask is filled with greenish yellow vapours of chlorine. After all the H_2SO_4 has been added, cock *F* is closed and the flask allowed to remain with gentle shaking from time to time for about 4–5 min. In this interval the chlorine is reabsorbed by the $\text{CrO}_3\text{-H}_2\text{SO}_4$ mixture with the probable formation of a chromic derivative which is not re-evolved on heating, and the flask clears. Cock *G* is now gradually opened and the contents of the flask digested as usual.

Organic and inorganic carbon. The method can be conveniently applied to the determination of organic and inorganic carbon (carbonates) in the soil by the determination of total carbon on one sample and of carbonates on another [Schollenberger, 1930]. A preliminary removal of carbonates by the action of dilute H_2SO_4 or phosphoric acid followed by the estimation of organic carbon by chromic digestion (keeping the ultimate H_2SO_4 : water ratio at the optimum point) has also been found to give concordant results.

The values for carbon obtained by this method for a selection of the large number of pure organic compounds, soils and plant materials examined by this method are given in Table I and show satisfactory agreement with the dry combustion values.

Table I. *Recoveries of carbon and nitrogen by chromic digestion.*

Soils	Materials	% carbon		% total N recovered as		
		Dry combustion	Chromic digestion	$\text{NH}_4\text{-N}$	$\text{NO}_3\text{-N}$	Gaseous N (lost)
1.	Arnish moor soil*	53.0	53.1	91	4.5	4.5
2.	Insch soil, Auchinbradie*	5.15	5.18	90	5	5
3.	Bangor soil*	3.15	3.11	85	7.5	7.5
4.	Hungarian Brown Chernozem†	2.36	2.29	90	Nil	10
5.	Ohehe tropical forest soil†	2.74	2.88	90	5	5
6.	Barmers Australian chloride soil, 4.6% Cl_2 †	0.53	0.56	65	35	Nil
7.	Ohio leaf mould layer†	17.83	18.01	95	2.5	2.5
8.	Hungarian Solonetz†	1.86	1.81	90	10	Nil
Plant materials						
1.	Rice straw	32.6	32.2	90	10	Nil
2.	Bracken leaves	44.7	45.0	90	10	Nil
3.	Grass (lawn mowings)	38.4	38.6	87.5	7.5	5
4.	Rape seed cake	40.0	40.4	90	5	5
Proteins						
1.	Egg albumin	42.1	42.4	90	5	5
2.	Caseinogen	43.6	43.2	92.5	2.5	5
3.	Blood albumin	43.2	43.0	90	5	5
4.	Edestin	—	—	82.5	7.5	10
5.	Gelatin	—	—	90	4	6
6.	Glutenin	—	—	92.5	3	4.5
7.	Haemoglobin	—	—	91	3	6
8.	Fibrin (blood)	—	—	90	4	6
9.	Keratin	—	—	92.5	3	4.5
10.	Peptone	—	—	88	4	8

* Soils from the A.E.A. Co-operative Analyses Series.

† Soils from the I.S.S.S. Organic Carbon Series, 1935.

The figures for carbon in soils by Dry Combustion are taken from: Report of Organic Carbon Committee, *Trans. of the Third Int. Congress of Soil Science*, 1, 1935; Report of Analysis of Soils Sub-Committee, *Agric. Progress*, 11, 1934.

ESTIMATION OF NITROGEN.

The residues after digestion with $\text{CrO}_3\text{-H}_2\text{SO}_4$ mixture yield low results for nitrogen, as compared with the Kjeldahl values, because a portion of the organic nitrogen is oxidised to nitrate. Added ammonium salts are quantitatively recovered under these conditions, showing that the phenomenon is not one of oxidation of ammonia by chromic acid. A more detailed examination of the course of oxidation of organic nitrogen in soils, plant materials, extracted proteins and simple organic substances has shown that of the total nitrogen in a substance a definite fraction is reduced to ammonia, the proportion depending on the structure of the substance. Of the fraction not reduced to ammonia a portion is oxidised to nitrate and the rest lost in gaseous form. These proportions of recovered ammonia expressed as percentages or fractions of total nitrogen have been defined as "oxidation constants" [Acharya, 1935]. Their values are fairly constant if amounts of the substances containing above 10 mg. of N in the case of soils and 20 mg. in the case of substances richer in nitrogen (*e.g.* proteins) are taken for oxidation. At lower levels a portion or the whole of the gaseous nitrogen which would otherwise be lost tends to be fixed as nitrate. The course of oxidation of pure organic substances will be dealt with in detail in a later communication.

The nitrogen values obtained by chromic digestion of some typical soils, plant materials and proteins are shown in Table I. It will be noted that in the case of soils and plant materials the recovery of nitrogen as ammonia is of the order of 90 % and hence an average correction of 10 % on the figure for direct distillation with alkali would give results agreeing within 1-2 % with the Kjeldahl figure. In the case of extracted proteins, however, there is more variation and this correction may not be found to be so satisfactory. It may be mentioned that in the case of organic compounds such as the purines, guanidine, iminazole and pyrimidine derivatives and others having two or more nitrogen atoms linked to the same C atom, the recovery of $\text{NH}_3\text{-N}$ is much lower and varies greatly from one compound to another. The application of a correction in such cases would obviously be of no value.

Influence of the amount of substance taken. This is shown for some typical soils and plant materials in Table II. For amounts of soil containing 5 mg./g. and less of nitrogen, the gaseous nitrogen fraction is oxidised to nitrate and hence the modified procedure to include nitrates (p. 246) gives results agreeing with the Kjeldahl figure. As the usual soil aliquot taken for carbon estimation corresponds to 50-75 mg. of carbon and the C : N ratio in most soils is about 10 : 1, the nitrogen values of the residues in most digested soil samples would be of the order of 5-7 mg., and the modified procedure to include nitrates would give results agreeing to within 98-100 % of the Kjeldahl figure.

Influence of chlorine. The interference of chlorine in the estimation of carbon has been noted already. It was found that chlorine interferes also in the estimation of nitrogen in that it increases the amount of nitrate formed at the cost of ammonia (Table III). The low values obtained in presence of chlorine have been attributed [Anderson and Schutte, 1924] to the liberation of free nitrogen according to the equation



or to the interaction of hypochlorous acid formed with ammonia [Subrahmanyam *et al.*, 1934]. The quantitative recovery of ammonium salts in presence of added chlorine under the experimental conditions shows, however, that the action of chlorine is on the amino-groups of protein rather than on the ammonia formed.

Table II. *Influence of the amount of substance taken on nitrogen recovery.*

Material	Amount of substance taken g.	Total N by Kjeldahl mg.	After chromic digestion			
			NH ₃ -N mg.	NO ₃ -N mg.	Total N recovered mg.	Gaseous N (lost) mg.
1. Inch soil, Auchinbradie	1	3.8	3.5	0.3	3.8	Nil
	1.5	5.7	5.2	0.5	5.7	Nil
	2	7.5	6.8	0.6	7.4	0.1
	3	11.3	10.2	0.7	10.9	0.4
	4	15.0	13.5	0.8	14.3	0.7
2. Arnish Moor soil	5	18.8	17.0	0.9	17.9	0.9
	0.2	3.8	3.5	0.3	3.8	Nil
	0.3	5.7	5.2	0.5	5.7	Nil
	0.4	7.6	6.9	0.5	7.4	0.2
	0.5	9.5	8.6	0.5	9.1	0.4
3. Grass (lawn mowings)	0.6	11.4	10.3	0.5	10.8	0.6
	0.10	3.6	3.2	0.4	3.6	Nil
	0.15	5.4	4.8	0.6	5.4	Nil
	0.2	7.2	6.4	0.6	7.0	0.2
	0.3	10.8	9.5	0.7	10.2	0.6
4. Rape seed cake	0.5	18.0	15.8	1.0	17.2	1.2
	0.1	5.6	5.2	0.4	5.6	Nil
	0.2	11.3	10.2	0.6	10.8	0.5
	0.3	17.0	15.2	1.0	16.2	0.8
5. Bracken leaves	0.4	22.6	20.2	1.2	21.4	1.2
	0.2	3.8	3.5	0.3	3.8	Nil
	0.4	7.6	6.9	0.7	7.6	Nil
	0.5	9.5	8.6	0.9	9.5	Nil
6. Egg albumin	0.6	11.4	10.3	0.9	11.2	0.2
	0.03	3.7	3.4	0.3	3.7	Nil
	0.05	6.2	5.6	0.6	6.2	Nil
	0.10	12.3	11.1	1.2	11.7	0.6
	0.20	24.4	22.1	1.2	23.3	1.1
	0.30	36.6	33.1	1.8	34.9	1.7

Table III. *Influence of chlorine on the recovery of nitrogen.*

(Figures expressed as percentages of total N.)

Material	No KCl added		0.1 g. KCl added		0.2 g. KCl added	
	% NH ₃ -N	% NO ₃ -N	% NH ₃ -N	% NO ₃ -N	% NH ₃ -N	% NO ₃ -N
1. Egg albumin	90	5	87.2	7.6	84.3	10.4
2. Caseinogen	92.5	2.5	90.6	4.1	88.6	6.2
3. Rice straw	90	10	87.4	12.4	85.5	14.2
4. Rape seed cake	90	5	87.7	7.1	84.7	10.1
5. Ohio leaf mould	95	2.5	88.7	8.6	82.8	14.4
6. Inch soil, Auchinbradie	90	5	83.5	11.2	74.4	20.2

Estimation of nitrate in presence of chromic and sulphuric acids. As other substances besides nitrate, *e.g.* hydroxylamine, yield ammonia on treatment with reducing agents, it seemed desirable to show the formation of nitrate and if possible to estimate it in the oxidising mixture by a more specific reaction. The amount of nitrate formed being usually of the order of 0.5–1 mg. in presence of 40–60 g. of H₂SO₄ and 3–5 g. of CrO₃, the phenoldisulphonic acid method of estimation was examined.

The method finally worked out was as follows. The digested residue (filtered through glass wool if necessary) is made up to a volume of 500 ml. or 1000 ml. (depending on the concentration of sulphuric and chromic acids) in a tall beaker.

It is warmed to 50° and the excess of CrO_3 reduced by Na_2SO_3 (solid) added little by little, testing externally with starch-iodide indicator. The liquid is rendered alkaline by the addition of solid CaCO_3 followed by $\text{Ca}(\text{OH})_2$, stirred well, allowed to settle and an aliquot of 50 ml. or 100 ml. of the clear supernatant liquid pipetted off for evaporation and estimation of nitrate in the usual way [Assoc. Off. Agric. Chem., 1930].

The method was found to give quantitative recoveries of added nitrate and the results with soils and plant materials agreed with those obtained by the Devarda method.

SUMMARY.

1. A procedure is described for the estimation of carbon and nitrogen in soils, plant materials and organic compounds by the action of a mixture of chromic and sulphuric acids under reduced pressure.

2. The results for nitrogen in soils and plant materials are too low on account of one portion being oxidised to nitrate and another lost in gaseous form. Where, however, the aliquot taken contains about 5 mg. or less of nitrogen the gaseous portion is also fixed as nitrate and correct figures are obtained by the estimation of the ammoniacal and nitrate-nitrogen present. For amounts higher than 5 mg. an average correction of 10% on the ammoniacal nitrogen formed gives results agreeing to within 98–100% of the Kjeldahl figure.

3. A procedure is described for the estimation of small amounts of nitrate in presence of large quantities of sulphuric and chromic acids.

The author's thanks are due to Sir E. J. Russell and Mr E. H. Richards for their interest in the work.

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XXXIX. A NEW SYNTHESIS OF METHIONINE AND A SCHEME RELATING CERTAIN α -AMINO-ACIDS.

By EVELYN MARY HILL AND WILLIAM ROBSON.

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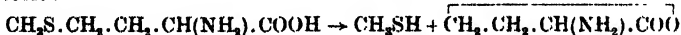
(Received December 11th, 1935.)

THE constitution of methionine has been fully established both by analysis [Barger and Coyne, 1928] and by synthesis [Barger and Coyne, 1928; Windus and Marvel, 1930; Barger and Weichselbaum, 1931]. Moreover, the process whereby it was originally isolated by Mueller [1923] from protein hydrolysates has been so modified [Pirie, 1932; 1933; Hill and Robson, 1934] that it is now one of the more readily accessible of the amino-acids.

The synthesis of methionine about to be described is of interest in that it reveals a possible relationship between certain of the known α -amino-acids, to which, so far as we are aware, attention has not previously been drawn.

The new synthesis originated in the observation that during Kjeldahl analysis of methionine there occurred a vigorous evolution of gas having the odour of a volatile sulphur compound. Since the odour was noticeable soon after heat was applied to the reaction mixture, it was thought that the sulphur compound resulted from a comparatively simple reaction and that it was probably dimethyl disulphide, formation of the latter arising by oxidation of the methyl mercaptan liberated from the methionine.

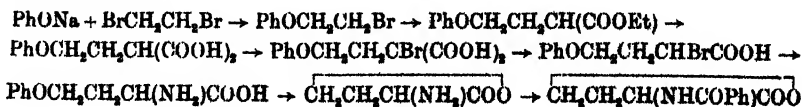
Such a non-hydrolytic liberation of methyl mercaptan from methionine appeared to us necessarily to involve simultaneous formation of α -amino- γ -butyrolactone:



At a later stage of our work we learned in fact that Butz and du Vigneaud [1932], in discussing the breakdown of methionine under the action of hot 18*N* H_2SO_4 , concluded that it was split partly into methyl mercaptan and α -amino- γ -hydroxybutyric acid and partly into methyl alcohol and homocysteine, from the oxidation of which latter compound homocystine arose. In this connection it should be added that, from the work of Fischer and Blumenthal [1907], who synthesised both α -amino- γ -hydroxybutyric acid and α -amino- γ -butyrolactone, it appears that the former compound is incapable of existing in acid solution since it reverts to the lactone in such a medium. In any case, it is an open question whether the decomposition, as we regard it, necessarily involves a molecule of water.

A further study of the probable course of the reaction raised the interesting question of its possible reversibility; in other words can methionine be synthesised from methyl mercaptan and α -amino- γ -butyrolactone?

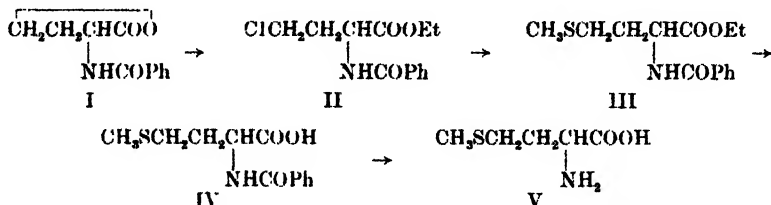
Selecting α -benzamido- γ -butyrolactone as the most suitable starting-point, we synthesised this compound according to the method of Fischer and Blumenthal [1907] as follows:



the only modifications being the preparations of (1) phenoxyethyl bromide in quantity by the admirable method of Marvel and Tanenbaum [1933] and of (2) ethyl phenoxyethylmalonate as described by Leuchs [1911].

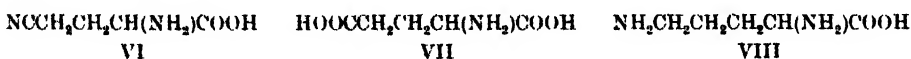
We first attempted to synthesise methionine by direct combination of methyl mercaptan with α -benzamido- γ -butyrolactone, but in this direction we have so far been unsuccessful. An alternative route however was available and the success attained along these lines makes it appear that a direct synthesis such as we have suggested above may be possible.

Treatment of a suspension of α -benzamido- γ -butyrolactone (I) in absolute alcohol with a rapid stream of dry hydrogen chloride gave ethyl α -benzamido- γ -chlorobutyrate (II),



an oil which gradually solidified on standing. The latter compound, dissolved in absolute alcohol, immediately reacted with a hot alcoholic solution of sodium methyl mercaptide, as evidenced by the separation of sodium chloride, to give the ester of benzoylmethionine (III), which in turn, on alkaline hydrolysis, gave benzoylmethionine (IV) in good yield and excellent purity. The removal of the benzoyl group by boiling hydrochloric acid completed the synthesis.

The synthesis of methionine from α -amino- γ -butyrolactone raises several questions. One of the more interesting of these is whether the aminolactone is the immediate precursor of methionine in nature. In this connection moreover we cannot overlook the fact that the lactone is closely related chemically to several of the naturally occurring α -amino-acids. On the one hand, it is, for instance, but a short step to the



lactone from aspartic acid, shown by Dunn and Fox [1933] to be derivable from fumaric acid. On the other hand the basic reaction whereby α -butyrolactone is converted into γ -cyanobutyric acid may be regarded as a possible basis of the conversion of the aminolactone into α -amino- γ -cyanobutyric acid (VI) from which one might proceed either by hydrolysis to glutamic acid (VII) or by reduction to ornithine (VIII), the latter compound being the direct precursor of arginine. The relationship might be extended in other directions but it will suffice to trace but one more connection at this point. Recently Garforth and Pyman [1935] have shown that α -amino- γ -butyrolactone is an excellent starting-point for the synthesis of substituted glyoxalines and it is conceivable that the natural synthesis of histidine may lie along some such path.

The main objection at the present time to the scheme which we have advanced above lies in the fact that neither α -amino- γ -butyrolactone nor α -amino- γ -hydroxybutyric acid has been found in nature either in the free state or as part of a larger molecule. Nevertheless, their relationship to other amino-acids is so close that we believe that a search for them might profitably be undertaken especially among plant products. In this connection it may be added that

Schryver and Buston [1926] reported the isolation of a hydroxylated α -amino-butyric acid from the acid hydrolysate of "oat-protein", but it was apparently not identical with the α -amino- γ -hydroxybutyric acid of Fischer and Blumenthal to which the authors make reference in their communication.

For purposes of record, the carbamido- and hydantoin derivatives of methionine have been made and their melting points and solubilities determined.

EXPERIMENTAL.

Ethyl α -benzamido- γ -chlorobutyrate. Through a solution of α -benzamido- γ -butyrolactone (10 g.) (prepared according to Fischer and Blumenthal with the modifications mentioned above) in absolute alcohol (150 ml.) was passed a rapid stream of hydrogen chloride until the temperature, which rose during the preliminary stage of the reaction, began to fall. Next morning the mixture was concentrated under reduced pressure and the residue extracted with dry ether. The insoluble portion was again esterified, the reagents removed as before, and the residue extracted with ether. The ethereal extracts were combined, the ether evaporated, and the residual oil poured into a crystallising basin where it rapidly solidified. The product melted at 45° and weighed 10.2 g. (78 % of theory).

Benzoylmethionine ethyl ester and benzoylmethionine. Methyl isothiocarbamide sulphate (6 g.) prepared according to the method of Arndt [1921] was hydrolysed with 5*N* NaOH (20 ml.) and the methyl mercaptan evolved directly absorbed in a solution of sodium (0.6 g.) in absolute alcohol (20 ml.). To the solution of the mercaptide was added a solution of ethyl α -benzamido- γ -chlorobutyrate (5 g.) in absolute alcohol (15 ml.) and the mixture refluxed for 4 hours. Next morning the alcohol was distilled off and water added to the residue; needles immediately commenced to separate. When crystallisation was completed, the solid (1.6 g.) was removed at the pump and the filtrate acidified. A further precipitate (2.4 g.) was thrown down and was seen to consist of long narrow plates. Examination of the two sets of crystals showed that the former consisted of ester, the latter of acid, partial hydrolysis of the ester apparently having occurred owing to the presence of the slight excess of sodium mercaptide in the reaction mixture.

The ester, without further purification, was hydrolysed with boiling 2 % NaOH (40 ml.) and the acid obtained on acidifying the cold hydrolysate added to that already isolated. The total yield of benzoylmethionine was 3.4 g. (72 % of theory). A portion of the product recrystallised from water melted at 151° (Windus and Marvel give $143\text{--}145^\circ$). (Found: N, 5.50 %. $\text{C}_{12}\text{H}_{16}\text{O}_3\text{NS}$ requires N, 5.54 %.)

Methionine. Benzoylmethionine (1.8 g.) was refluxed with 20 % HCl (50 ml.) for 18 hours. The hydrolysate when cold was extracted with ether to remove benzoic acid and the aqueous solution concentrated *in vacuo* almost to dryness. The residue was dissolved in absolute alcohol and the solution made just alkaline to bromophenol blue with pyridine. On standing for 48 hours the methionine separated. This was removed at the pump, washed with alcohol and ether and dried. It weighed 0.6 g. (55 % of theory). (Found: N, 11.85 %. $\text{C}_5\text{H}_{11}\text{O}_2\text{N}$ requires N, 11.96 %.)

Carbamidomethionine. Methionine (4 g.) was heated on a water-bath with a solution of potassium cyanate (2.7 g. = 1.25 mols.) in water (50 ml.). When cold, the solution was neutralised with HCl and evaporated to dryness under reduced pressure. The residue was extracted with rectified spirits, the extract being

again evaporated to dryness under reduced pressure. A portion of the carbamido-acid so obtained (the yield was theoretical) was recrystallised from water, after which it melted at 171–172°. Carbamidomethionine is very soluble in water and alcohol but insoluble in ether.

Methionine hydantoin. Carbamidomethionine (3 g.) was boiled with dilute HCl (10 ml.) for 5 min. and the solution evaporated to a small volume and cooled. Methionine hydantoin crystallised out. The solid was removed at the pump. A further crop of the hydantoin was obtained by concentrating the filtrate. The product (2.5 g.) after recrystallisation from water melted at 117–118°. (Found: N, 16.00%. $C_6H_{10}O_2N_2S$ requires N, 16.09%.)

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XL. THE DETERMINATION OF PHYTIC ACID.¹

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(Received December 14th, 1935.)

THE method generally accepted for the determination of phytic acid (*i.e.* inositol-hexaphosphoric acid) is that of Heubner and Stadler [1914]. It was subsequently applied to plant products and foodstuffs by other workers [Rather, 1917; Arbenz, 1922; Averill and King, 1926] and Rather [1917] found that its accuracy is not affected by non-phytic acid substances in plant extracts. The method involves the titration of phytic acid in 0.6 % HCl solution with standard FeCl_3 (0.5–2.0 mg. Fe per ml.) in 0.6 % HCl, ammonium thiocyanate being used as internal indicator; Heubner and Stadler considered that the end-point is reached when a flesh pink colour is obtained which persists for 5 min.

A disadvantage of this method is that the end-point is not sharp owing to the presence of the colloidal precipitate of ferric phytate which forms during titration. Various attempts have been made to circumvent this difficulty [Knowles and Watkin, 1932; Andrews and Bailey, 1933], the most satisfactory method being that of Harris and Mosher [1934] who titrated past the end-point, filtered the precipitate and determined the excess FeCl_3 colorimetrically.

Phytic acid is determined in biological material by extraction with dilute HCl, the titration being performed directly on the extract. This procedure gives rise to a serious difficulty as it cannot be applied to materials which yield markedly coloured extracts.

Experiments by the author showed that phytic acid is partially adsorbed on substances such as norite charcoal, fuller's earth and alumina and therefore decoloration of the extracts by such adsorbents cannot be used as a preliminary to titration. It was found that solutions of FeCl_3 in *N/6* HCl did not yield precipitates at room temperature with very dilute solutions of phytic acid in *N/6* HCl. When heated at 100° however coagulated precipitates formed which were easily separable either by centrifuging or filtration. Under suitable conditions precipitation was complete and furthermore it was possible to decompose the precipitates quantitatively with NaOH into $\text{Fe}(\text{OH})_3$ and sodium phytate. By using centrifuging for separating the ferric phytate precipitate it became possible to deal with small amounts of phytic acid and in order to take advantage of this fact a micro-method for the determination of phytic acid was developed. It was designed to determine up to 2 mg. (expressed as P) of phytic acid.

I. THE COLORIMETRIC DETERMINATION OF PHYTIC ACID.

This method differs from previous methods mainly in that (1) a fixed amount of standard FeCl_3 is added to the solution being analysed, (2) the ferric phytate is precipitated at 100°, (3) the precipitation is performed in *N/6* HCl and (4) the excess Fe^{+++} after precipitation of the ferric phytate is determined by the thiocyanate method using a colorimeter.

Conditions of precipitation. The precipitations are done in boiling-tubes heated in the water-bath in racks. Experiments showed that a solution of 2.5 mg.

¹ A preliminary account of the work described in this paper was the subject of a paper read at the Biochemical Society meeting held on March 15th, 1935 [Young, 1935].

of Fe^{+++} in 5 ml. of N HCl was suitable for the precipitation of quantities of phytic acid up to 2 mg. (expressed as P) in 25 ml. of neutral or slightly acid solution. Under these conditions the required final concentration of $N/6$ HCl was obtained. After the other details of the method had been established a series of time experiments was performed in order to find the effect of the period of heating on the precipitation process. The following results were obtained:

mg. of phytic acid P taken	mg. of Fe^{+++} precipitated after a heating period of			
	5 min.	10 min.	15 min.	30 min.
0.20	0.14	0.17	0.20	0.19
0.50	0.54	0.53	0.53	0.53
1.00	1.07	1.08	1.07	1.06
1.50	1.57	1.57	1.57	1.58
2.00	2.04	2.03	2.03	2.03

Phosphoric acid is only liberated with difficulty when phytic acid is subjected to hydrolysis and the fact that decreasing values were not obtained with prolongation of the time of heating in the above experiments shows that no significant amount of hydrolysis occurs during the precipitation process. In the case of 0.2 mg. of phytic acid-P marked coagulation of the precipitate did not occur in less than 15 min. heating and since this period also represented the maximum precipitation of Fe^{+++} for this amount of phytic acid it was adopted for use in the method.

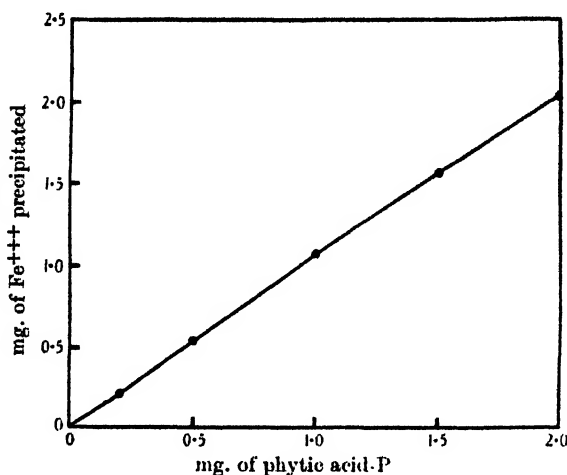
The determination of excess Fe^{+++} . The colorimetric thiocyanate method appeared to be the most suitable means of determining the excess Fe^{+++} , and Brouckère and Gillet [1933] have shown that this procedure can be applied with accuracy to solutions containing HCl up to N concentration.

✓ The ferric phytate precipitation is performed in $N/6$ HCl solution (30 ml.), and after the precipitation the volume of the reaction mixture is made up to 50 ml. with $N/6$ HCl and then filtered. If 20 ml. of this filtrate are taken the maximum amount of the excess Fe^{+++} can be 1.0 mg. An investigation was therefore made of the determination of Fe^{+++} in quantities up to 1.0 mg. Using a standard containing 2.0% KCNS and 0.5 mg. of Fe^{+++} in 50 ml. of $N/6$ HCl it was possible to determine accurately amounts of Fe^{+++} between the limits 0.25–1.0 mg.

Quantitative results. Sodium phytate was prepared from a sample of commercial calcium magnesium inositolhexaphosphate ("Phytin" S.C.I., Basle) by the method of Posternak [1921]. The product was recrystallised twice and air-dried. It contained no detectable amounts of inorganic P and gave the following analyses: Na, 17.11%; total P, 11.41%; phytic acid-P (by the method of Heubner and Stadler), 11.48%; H_2O lost at 105–110°, 39.28%; $\text{Na}_{12}\text{C}_6\text{H}_6\text{O}_{24}\text{P}_6 \cdot 3\text{H}_2\text{O} + 35\text{H}_2\text{O}$ requires Na, 17.16%; total P, 11.57%; H_2O (calculated for $35\text{H}_2\text{O}$), 39.18%. Standard solutions were prepared from the crystalline sodium phytate. They were made just acid to litmus with HCl and were used for determining the amounts of Fe^{+++} precipitated in the colorimetric method by varying amounts of phytic acid. Blank experiments using water were also performed. The mean results obtained were as follows:

mg. of phytic acid P taken	mg. of Fe^{+++} found	mg. of Fe^{+++} precipitated
None	2.50	None
0.20	2.30	0.20
0.50	1.97	0.53
1.00	1.43	1.07
1.50	0.93	1.57
2.00	0.47	2.03

The graphical representation of these results shows that a relationship closely approaching a linear form exists between the varying amounts of phytic acid and the corresponding amounts of Fe^{+++} precipitated.



(Graph showing ferric iron-phytic acid-P relationships in the colorimetric method.

Reagents. The following reagents are required for the estimation:

Standard FeCl_3 in N HCl solution. A solution of A.R. FeCl_3 in N HCl is made up and Fe^{+++} determined gravimetrically. By suitable dilution with N HCl a solution containing 0.5 mg. of Fe^{+++} per ml. is prepared.

$N/2$ and $N/6$ HCl.

A 10% solution of A.R. KCNS in water.

Procedure. Into a dry pyrex boiling-tube are pipetted 25 ml. of the solution for analysis (neutral or just acid to litmus), and 5 ml. of the standard FeCl_3 -HCl solution are added. The tube is covered by a glass bulb and heated in a rack in a boiling water-bath for 15 min. with the level of the water above that of the contents of the tube. The ferric phytate separates as an ivory coloured flocculent precipitate. After cooling for 15 min. in a bath of cold water the contents of the tube are made up to 50 ml. with $N/6$ HCl. The contents of the flask are filtered into a dry boiling-tube through a dry 9.0 cm. no. 31 Whatman filter. 20 ml. of the filtrate are pipetted into a 50 ml. flask (in cases where the amount of phytic acid approaches the upper limit determinable by the method the small excess of iron makes it preferable to use 30 ml. instead of 20 ml. of this filtrate), 5 ml. of $N/2$ HCl and 10 ml. of 10% KCNS solution are added, the solution is made up to 50 ml. with $N/6$ HCl, mixed and compared without delay in a colorimeter with the standard. The standard is prepared as follows: 5 ml. of the standard FeCl_3 -HCl solution in a 50 ml. flask are diluted with 25 ml. of water and made up to 50 ml. with $N/6$ HCl. 10 ml. of this solution are pipetted into a 50 ml. flask, 5 ml. of $N/2$ HCl and 10 ml. of 10% KCNS solution are added and the volume is made up to 50 ml. with $N/6$ HCl. The most convenient colorimeter reading for the standard is 20 (2.0 cm.), and by using this reading and 20 ml. of the filtrate for the determination of excess Fe^{+++} , the mg. of Fe^{+++} precipitated by the phytic acid can be calculated from the expression $2.50 - \frac{\text{Reading of unknown}}{25}$.

From the amount of Fe^{+++} precipitated the quantity of phytic acid P is obtained by reference to the graph. For conversion of the results for phytic acid-P into phytic acid ($\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$) the factor is 3.55.

II. THE DETERMINATION OF PHYTIC ACID IN FAECES.

In studying the metabolism of phytic acid in rabbits [Young *et al.*, 1935] it became necessary to determine phytic acid in faeces. Since this material yields coloured extracts, the following separation procedure was evolved. The phytic acid is precipitated from the extract with FeCl_3 in dilute HCl solution, the precipitate is separated by centrifuging, decomposed with NaOH , the $\text{Fe}(\text{OH})_3$ removed by filtration and the filtrate (containing sodium phytate) used for the determination by the colorimetric method. The various stages of this procedure are considered in detail below.

Extraction of the phytic acid. Most recent workers have used 2% HCl for extracting phytic acid from biological material, *e.g.* Harris and Mosher [1934] used 25 ml. of 2% HCl for each g. of material and extracted for 3 hours with occasional shaking. In the present work it was found more convenient to use $N/2$ HCl for extraction. Repeated extraction showed that a single extraction under conditions to be described is sufficient.

Precipitation of ferric phytate. Previous workers have reduced the acidity of the extract to 0.6% HCl by dilution and then proceeded to titrate the phytic acid in this solution. In the present method the extract is first neutralised, made just acid to litmus with HCl and then filtered. To a measured volume of the filtrate is added one-fifth of its volume of a FeCl_3 solution in N HCl and the required acidity is thus obtained. This procedure has the advantage that it avoids a large increase in the volume of the extract and also brings about the precipitation and removal of much unwanted material from the extract preliminary to the ferric phytate precipitation. With 20 ml. of slightly acid extract (containing not more than 4 mg. of phytic acid-P) it was found necessary to use 4 ml. of a N HCl solution of FeCl_3 containing 1.25 mg. Fe per ml.

Determination of the phytic acid of the precipitate. The precipitate is suspended in about 10 ml. of hot water in the centrifuge-tube and then converted into sodium phytate and ferric hydroxide by the action of sodium hydroxide. By calculation and experiment 2 ml. of $N/2$ NaOH were found to be adequate for the treatment of precipitates containing up to 4 mg. of phytic acid-P. The ferric hydroxide coagulates and is removed by filtration and washed with hot water. The filtrate and washings are made just acid to litmus with HCl . In the case of a faeces determination the solution obtained at this point is colourless and also gives no colour with KCNS . The phytic acid content of this final solution is determined by the colorimetric method described above (I).

Quantitative results. After the above conditions had been established, the efficiency of the separation procedure was tested using slightly acid solutions containing 1, 2, 3 and 4 mg. of phytic acid-P. The following results were obtained.

mg. of phytic acid-P taken	mg. of phytic acid-P found	
1.00	0.96	0.98
2.00	1.98	1.96
3.00	2.94	2.94
4.00	3.92	3.92

The average recovery in these 8 experiments was 97.9%.

The method has the advantage that it eliminates interference by inorganic phosphate, *e.g.* a solution containing 1.25 mg. phytic acid-P gave a recovery of 1.20 mg. whereas a similar solution containing in addition 15 mg. of inorganic P (present as Na_2HPO_4) gave a recovery of 1.21 mg.

The method was tested with extracts of rabbit faeces. In each experiment two equal volumes (A and B) of the $N/2$ HCl extract were taken and a known amount of phytic acid added to B. The determinations were then performed as described in detail below. The results were as follows:

mg. of phytic acid-P			
Added to B	Found in A	Found in B	Difference
0.50	0.58	1.07	0.49
0.50	0.50	1.00	0.50
1.00	0.72	1.68	0.96
1.00	0.78	1.72	0.94
1.00	0.68	1.63	0.95
1.00	0.68	1.64	0.96
1.00	0.84	1.83	0.99
1.00	1.31	2.24	0.93
1.00	1.28	2.25	0.97
1.00	1.07	2.03	0.96

The average recovery of added phytic acid in this series of experiments was 96.4 %.

No phytic acid was found when the method was applied to the faeces of rabbits fed for some days on a diet (cabbage) free from phytic acid thus showing that under these conditions the faeces contained no interfering substances.

Reagents. The following reagents are required.

FeCl_3 in N HCl solution. A solution of A.R. FeCl_3 in N HCl is prepared containing 1.25 mg. Fe^{+++} per ml.

$N/2$ HCl.

5*N* and $N/2$ NaOH solution.

Procedure. The faeces are dried, sieved and extracted by mechanical shaking for 2 hours in a glass-stoppered bottle with 25 ml. of $N/2$ HCl per g. of material. The extract is filtered on a Büchner funnel and 40 ml. of the filtrate are transferred to a 50 ml. flask, neutralised with 5*N* NaOH, made just acid to litmus with HCl, made up to 50 ml. with water and filtered. A measured volume of the filtrate (containing not more than 4 mg. of phytic acid-P) is transferred to a centrifuge-tube and one-fifth of its volume of the FeCl_3 -HCl solution is added. The tube and its contents are heated in a boiling water-bath for 15 min., cooled in a bath of cold water for 15 min. and centrifuged for 10 min. at 3000 r.p.m. The supernatant fluid is decanted and the tube is allowed to drain. The precipitate is suspended in 10 ml. of hot water in the centrifuge-tube and heated in a boiling water-bath for 2 min. To the hot suspension 2 ml. of $N/2$ NaOH are added with stirring, and heating in the water-bath is continued for 15 min. with occasional stirring. The precipitate of $\text{Fe}(\text{OH})_3$ is filtered on a 7 cm. no. 31 Whatman filter and washed with hot water. The filtrate and washings are allowed to cool, made just acid to litmus with HCl and the volume made up to 50 ml. with water. 25 ml. of this solution are used for the determination of the phytic acid by the colorimetric method.

CONCLUSIONS.

Although a detailed study of this procedure has only been made in the case of faeces it seems probable that it may prove of value in determining phytic acid in other biological materials. It minimises the action of interfering substances.

and can be applied to materials yielding coloured extracts, which is not possible with the direct titration methods previously used. Furthermore the colorimetric method permits the accurate determination of smaller amounts of phytic acid than has been hitherto possible.

SUMMARY.

A description is given of a colorimetric method for determining phytic acid in amounts up to 2 mg. (expressed as P). A procedure is also described which has been developed to permit the determination of phytic acid in biological materials which yield coloured extracts and an account is given of its application to faeces.

The author is indebted to Prof. J. C. Drummond for his interest and advice during the course of this work.

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XLI. BIOCHEMICAL STUDIES IN THE NITROGEN METABOLISM OF THE APPLE FRUIT.

II. THE COURSE FOLLOWED BY CERTAIN NITROGEN FRACTIONS DURING THE DEVELOPMENT OF THE FRUIT ON THE TREE.¹

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WHILST the carbohydrate, pectin and acid metabolism of the apple fruit during its growth on the tree, and of the detached fruit placed at various constant temperatures in darkness, have been closely studied, the nitrogen metabolism has received little detailed attention.

The results of the work on the metabolism of carbon make it clear that there are three physiologically important stages in the development of the apple. Firstly, meristematic cell-division ceases at an early stage in development—when the fruit weighs only 1 or 2 g. Secondly, there is a sharp maximum in the curves representing the concentration of starch during August, the exact date of this maximum varying slightly in different varieties of apples. Thirdly, the concentration of sugars, which is low in the very young fruits, rises to a maximum as the fruit matures.

It is obviously important to ascertain whether any such definite stages occur in the metabolism of nitrogen in the fruit.

The first recorded attempt to determine changes in the constituent groups of the total nitrogen is that of Archbold [1925], who confined her attention to mature detached fruits. This worker was unsuccessful in her efforts to isolate an amount of soluble nitrogen sufficient for the estimation of the fractions thereof and came to the conclusion that the nitrogenous substances present in the apple fruit are all of a protein nature, a small fraction of this protein being water-soluble. Pilling and Pearsall [1930], in work of a preliminary nature, were able to obtain appreciable amounts of non-protein-nitrogen soluble in 60–80 % alcohol from mature apple fruits.

EXPERIMENTAL.

1. *Material and methods of sampling.*

Preliminary work has indicated that it is essential to devote considerable attention to the sampling of the material and to its preparation for analysis, as well as to the methods of chemical analysis to be employed. Duplicate samples must be prepared and analysed at intervals during the course of an experiment so that a statistical treatment of results becomes possible.

¹ The major part of this paper has been abstracted from a thesis accepted for the degree of Ph.D., Cambridge University, 1935.

The material and the method of sampling employed in the present work were, briefly, as follows.

Series HY/33. Fruit from 10 Bramley's Seedling trees on Stirling Castle stocks (18 years old) at Messrs Chivers' farm, Histon. Date of petal-fall,¹ May 15th, 1933. Firstly, each tree and each main branch of each tree were numbered; each apple and each main branch were also given a number, the first apple on each branch being number 1, the second number 2 and so on. Before each gathering the total number of fruits per tree to be gathered was decided. Next, the branch and the apple on each branch to be gathered were determined by a random selection of numbers. Any fruit obviously blemished or dropped during gathering was discarded, and the next in order up its branch was taken in its place. When two samples were gathered at once, alternate fruits were placed in each of two tins to give the two samples. Each sample was composed of not less than 25 fruits. The fruit was transferred to the laboratory in tins provided with well-fitting lids to minimise evaporation.

Series MBY/34. Fruit from 18 Bramley's Seedling trees (15 years) on East Malling stocks I and II on an unmanured plot at East Malling Research Station. Date of petal-fall, May 20th, 1934. Method of sampling exactly as for series HY/33.

Immediately on reaching the laboratory the fruit was weighed and halved, and the seeds were removed and weighed into sealed boiling-tubes. Fruit and seeds were then transferred to a room maintained at -20 to -25° to await preparation for analysis.

2. Preparation of material for analysis.

Owing to the time taken for analysis *etc.*, it became obvious that a method of preparation of the material for analysis must be chosen which would allow of at least several weeks of storage prior to analysis. The method finally adopted was that described by Onslow *et al.* [1927].

The advantages of this method of preparation and storage of the material are that it allows of:

- (1) Reasonably constant separation into peel and pulp.
- (2) Complete disintegration of the tissue.
- (3) Thorough mixing of the resultant frozen powder.
- (4) No change in the nitrogenous fractions in the tissue over periods of at least 2 months [Hulme, 1932].

Since, as will be seen later, soluble nitrogen is to be extracted with 60–80 % alcohol, this alcohol can be added to the frozen powder, so that no enzymic action is likely to occur during thawing of the tissue.

Separation of peel and pulp. The difficulty in any attempted separation of the peel and the pulp of the apple fruits is that while "true" peel-cells differ anatomically and chemically from "true" pulp-cells, the two tissues are contiguous, and merge into one another in the region of their juncture. The procedure found to give as good a separation as possible without special treatment (which might interfere with the distribution of nitrogen in the tissues) was to peel the apples while frozen hard with an ordinary domestic potato peeler set to a slightly smaller cutting angle than normal. Microscopic examination of the "peel" thus obtained, whilst showing, as would be expected, that a shallow layer of pulp was included, nevertheless indicated that a reasonably uniform separation could be achieved. It should be noted that the peel has a considerably higher

¹ "Petal-fall" refers to the stage when practically all the petals had shrivelled and fallen from the young apple fruits—the weight of the fruit at this stage was of the order of 0.2 g.

content of nitrogen than the pulp and differs considerably from it in the distribution of the various fractions of the nitrogen.

The values for the ratio pulp/peel for the two series of apples are shown in Table I. Care was taken to lose as little tissue as possible during peeling, and from the total weight of peel and of pulp for each sample (taken before grinding) the amount of each of these tissues per apple of the sample was calculated.

Table I.

Series ...		HY/33			MBY/34				
Sample	Days' development (from petal fall)	Av. wt./apple	Loss on peeling* (%)	Ratio pulp/peel	Sample	Days' development (from petal fall)	Av. wt./apple	Loss on peeling* (%)	Ratio pulp/peel
V	22	8.0	<1	2.81	I	19	3.2	4.1	2.30
VI (1)	29	18.6	2.8	4.95	II (1)	26	6.5	5.2	3.84
VI (2)	29	18.9	2.8	4.77	II (2)	26	6.5	4.0	4.01
VII	43	50.3	2.3	6.67	III	33	16.7	2.3	5.22
VIII	58	84.8	1.8	6.50	IVa (1)	40	26.2	3.2	5.52
IX (1)	67	113.2	1.4	6.96	IVa (2)	40	25.6	3.2	5.43
IX (2)	67	111.1	1.6	6.06	V	51	41.1	2.6	8.24
X	77	124.9	1.9	8.79	VIa (1)	60	49.7	3.8	7.02
XI (1)	93	168.4	2.0	8.75	VIa (2)	60	52.7	3.2	7.22
XI (2)	93	164.6	1.3	7.76	VII	73	71.8	4.0	7.03
XII	106	187.9	1.1	10.0	VIII	92	102.4	1.2	7.78
XIIIa (1)	124	219.0	1.1	11.1	IXa (1)	116	123.8	3.1	8.01
XIIIa (2)	124	218.5	1.0	11.0	IXa (2)	116	126.9	2.7	8.19
†XIIIb (1)	124	209.0	2.0	11.4	X	138	159.4	3.2	7.10
†XIIIb (2)	124	214.0	2.3	10.8	XIa (1)	150	157.6	2.8	8.18
XIV	141	245.6	1.7	12.5	XIa (2)	150	164.3	2.5	8.38
XV (1)	170	243.7	1.8	12.0	XIIa	163	172.7	1.8	8.14
XV (2)	170	240.0	2.0	11.8	XIIb	163	178.1	2.6	8.53
Average value 1.8					Average value 3.1				

* The inevitable loss of a small amount of tissue during peeling (see Table I, columns 4 and 9) causes a slight error in the calculations, but it is clear that this error is very small and reasonably constant.

† Samples for experimental storage not described in this paper.

3. Methods of analysis.

Determination of the total nitrogen content of the tissue.

Total nitrogen was determined on aliquot portions of the frozen, ground tissue by the usual macro-Kjeldahl method. Normally 50 g. of the pulp and 30–40 g. of the peel were taken for each determination. Despite the tendency for the digestion mixture to froth over during the early stages of heating it was found preferable not to dry the tissue before digestion, since this latter process was slow and gave erratic results. In agreement with the results of Pilling and Pearsall [1930], nitrates were found normally to be absent from the material, so that modifications of the Kjeldahl method to include these compounds were not used in routine determinations. In later work selenium was used [Lauro, 1931] in addition to copper sulphate as catalyst, thereby considerably reducing the time of digestion. The agreement obtained in these estimations is illustrated by the following results for six 50 g. sub-samples of a sample of pulp: average nitrogen content 0.0345%; standard deviation 0.00045%, average "blank" value on reagents alone, 0.0002%.

Separation of protein- and non-protein-nitrogen. In the present instance it was not desired, necessarily, to determine the protein-nitrogen directly, but to be certain of its separation from the simpler non-protein-nitrogen in order that a detailed investigation of the latter might be made. The method of attaining this object was based on the following facts established after a lengthy survey of the problem, using Bramley's Seedling apple fruits.

1. Water in the cold (with or without neutralisation of the free acid of the tissue as extraction proceeded), Mothes's tannin solution [Mothes, 1926] and 60–80% alcohol (under the conditions of the "standard" alcohol extraction method described below) extract equal amounts of nitrogen from apple pulp tissue. In the case of peel tissue small differences occur in the total extractable nitrogen which are reflected in the "precipitated" nitrogen fraction (see below). Such differences appear to be connected with the difference in solubility in water and in alcohol of the chlorophyll and associated pigments of the tissue.

2. Excluding the seeds, proteins directly soluble in water¹ or in 60–80% alcohol appear definitely to be absent from the apple fruit. In view of the work of Finn [1932] on the effect of freezing in relation to denaturation of proteins it cannot be argued with any certainty that the method of preparation of the tissue for analysis, involving as it does rapid freezing, is likely to lead to denaturation of protein with consequent loss of solubility in water. The results of Lincoln and Mulay [1929] for plant material also tend against such a possibility.

3. Extraction of the tissue with boiling 80% alcohol at atmospheric pressure gave a significantly lower result for total non-protein-nitrogen as compared with that obtained by the three methods described above.

4. After hydrolysis of a sample of apple pulp tissue with 25% hydrochloric acid, removal of excess acid, adjustment of the p_H of the hydrolysate to 3.5 (within the region of the p_H of fresh juice from Bramley's Seedling apples) followed by extraction of this hydrolysate with 70% alcohol exactly as in the "standard" method described below, 99% of the total nitrogen of the tissue was recovered in the extract finally obtained.

5. Hiller and Van Slyke [1922], working with blood, found that whilst 84% alcohol precipitated intermediate protein hydrolysis compounds fairly completely and consistently, about 27% of the free amino-acids present were adsorbed on the protein precipitates. These workers indicated, however, that this fraction (adsorbed amino-acids) "is sufficiently constant to validate conclusions drawn from comparable results in physiological experiments". In the case of apple tissue the similarity in the amount of nitrogen extracted by water and by alcohol makes it clear that there can be no appreciable adsorption of amino-acids on protein precipitated within the tissue by alcohol when the latter is used as extractant.

6. It is extremely difficult to attain a satisfactory separation of water extracts from the residual apple tissue. Filtration, with or without suction, is impossibly slow for routine work and centrifuging is little better. On the other hand alcoholic extracts filter readily. This difference is due, apparently, to the large amount of pectin present in the tissue, which, during water extraction, forms a slimy gel around the surface of the tissue.

In view of these considerations the following "standard" alcohol extraction method was used to obtain the non-protein-nitrogen fraction of the fruit tissue.

A suitable amount of tissue (300–500 g. in the case of pulp and 100–250 g. in the case of peel, depending on the amount of nitrogen present) was allowed to stand overnight at 12° with sufficient 97% alcohol to make the final alcohol concentration about 65%, assuming the moisture content of the tissue to be 85% of its fresh weight. The alcoholic extract (1st extract) was then filtered through a Soxhlet thimble of sufficient size to retain all the residual tissue. The tissue in the thimble was then extracted in a continuous vacuum extractor until extraction of soluble nitrogenous compounds was complete (2nd extract). This required 6–8 hours depending on the nitrogen content of the tissue. No attempt was made to neutralise the acids present in the tissues before or during extraction but the extractor was of such a form that the tissue only came into contact with alcohol at room temperature and the temperature of the extract either during extraction or during the subsequent evaporation of the alcohol never rose above 30°. The actual strength of the alcohol coming into contact with the tissue in the extractor was 85–87%. The 1st and 2nd extracts were combined, the alcohol was removed and the extracts were concentrated to a small volume *in vacuo*. The final alcohol-free extract was transferred to a standard flask and brought to a definite volume (60–150 ml. depending on the amount of nitrogen present). After bringing to volume the extract was filtered by suction through an asbestos pad. The residue on the pad was composed of com-

¹ It is interesting to note that Karmarkar [1934] found practically no water-soluble protein in the bark, wood and leaves of the Newton Wonder apple.

pounds which are precipitated during the removal of the alcohol (alcohol-soluble, water-insoluble compounds) and which appear to be chiefly chlorophyll and associated pigments. Its bulk was very small in the case of pulp tissue but larger in the case of peel. The nitrogen of this residue—designated “precipitated” nitrogen—was low and relatively constant (pulp 1–1.5% of the total nitrogen of the tissue; peel 2–3% of the total nitrogen).

Owing to the small amount of nitrogen present it was necessary to use micro-methods for the investigation of the nitrogen content of the extracts. The following are the fractions determined and the methods used in their determination.

1. *Total soluble nitrogen.* This refers to the total nitrogen of the extracts after removal of the “precipitated” nitrogen. A modification of the micro-Kjeldahl method as described by Pregl [1924] was used in this determination. An investigation of several methods of decreasing the time of the acid digestion led to the employment of hydrogen peroxide in this connection. As found by Christiansen and Fulmer [1927] for macro-Kjeldahl determinations on yeast, the judicious use of this reagent, apart from greatly accelerating the digestion, also gave results as much as 10% higher than those obtained by the normal digestion method. 2 ml. of 100 vol. hydrogen peroxide (B.D.H., N.A.R.) were found to give optimum figures for the nitrogen content using 1 ml. of a whole range of pulp and peel extracts. The method adopted was to add to 1 ml. of extract 1 ml. of hydrogen peroxide together with 2 ml. of sulphuric acid and heat gently for about 15 min. After cooling slightly a further 1 ml. of hydrogen peroxide and 3 ml. of sulphuric acid were added together with the usual amount of potassium sulphate and copper sulphate.¹ The digestion and subsequent steam-distillation were proceeded with as usual using *N*/70 acid with methyl red indicator to absorb the ammonia evolved in the latter process.

2. *Free ammonia- and amide-nitrogen.* Free ammonia was estimated as follows: 10 ml. of apple extract were just neutralised with 40% NaOH, 5 ml. of a 10% suspension of pure magnesium oxide were added and the ammonia liberated was distilled under diminished pressure at 40° into *N*/70 acid. The special micro-distillation apparatus used was the same as that used in the amide-nitrogen estimations. Amide-nitrogen was determined by the method used by Chibnall and Westall [1932]. 5 ml. lots of the apple extracts were used in this estimation. On employing the method suggested by Chibnall and Westall for the differential estimation of asparagine and glutamine with several extracts from mature apples, it became clear that glutamine was absent. The assumption was made, therefore, that all the amide-nitrogen originated from asparagine. The possibility of the existence of glutamine in immature apples was not investigated, but this compound is unlikely to be stable in so acid a medium as that of the Bramley's Seedling apple. Hence the *asparagine-nitrogen* = amide-nitrogen \times 2.

3. *Amino-nitrogen.*² This fraction was estimated on the residue from the ammonia-nitrogen estimation by the method already described [Hulme, 1935, 1]. Various modifications of the formaldehyde and alcohol titrations for the estimation of amino-acid groups were also investigated. It was found impossible to obtain satisfactory results with these methods chiefly owing to the large amount of colouring matter present. Richardson [1934] has also pointed out the advantages of the Van Slyke gasometric method for estimating amino-nitrogen in coloured biological liquids.

Amino-acid-nitrogen = amino-N – amide-N.

4. “*Rest*”-nitrogen = total soluble nitrogen – (free ammonia-N + asparagine-N + amino-acid-N).

5. “*Protein*”-nitrogen = total N – total soluble N.

Titrateable acid was also estimated in the apple extracts. 2–5 ml. of extract were titrated with 0.1 *N* NaOH, after dilution to 500 ml., using chlorophenol red ($p_K=6$) as indicator according to the suggestion of Bennet-Clark [1933]. Since the colouring matter present interfered somewhat with the end-point in these estimations—even after the dilution of the extracts—the end-points were checked with the Morton glass electrode. The titrateable acid figures given in the present paper were calculated as malic acid. All p_H values quoted were obtained by means of the Morton glass electrode.

¹ Since the above method was evolved it has been noted that Pregl [1930] now recommends the use of hydrogen peroxide to accelerate the micro-Kjeldahl digestion.

² This fraction is, of course, derived entirely from aliphatic amino-groups.

Statistical treatment.

Standard deviations were calculated from the following equations [Fisher, 1928].

Variance = $\Sigma d^2 / (\text{degrees of freedom} - \text{no. of means})$, d being the deviation of duplicates from their mean.

Standard deviation, $\sigma = \sqrt{\text{variance}}$.

The significant difference between two consecutive results in a series was obtained from the equation

$$\text{Sig. } D = \sigma \times \sqrt{2 \times t},$$

values of t ($P=0.05$) being obtained from Fisher's tables. In the many cases examined the standard deviation of sampling (for all the nitrogen fractions) was found to be 3 or 4 times as great as the standard deviation due to extraction alone (this latter of course also includes deviations due to experimental "error" of actual estimation), hence the former deviation only will be considered for each series. The vertical lines given to the right of the progress curves for each nitrogen fraction in the figures represent the magnitude of the significant differences calculated from this standard deviation.

RESULTS.

The results obtained are given in Figs. 1 to 9.

The following is a key to Figs. 2 to 8.

- | | | |
|---|------------------------|-----------------|
| ■ | Total nitrogen | } Series MBY/34 |
| ● | "Protein"-nitrogen | |
| ○ | Total soluble nitrogen | |
| □ | Amino-acid-nitrogen | |
| △ | Asparagine-nitrogen | |
| ▲ | "Rest"-nitrogen | |
| × | Free ammonia-nitrogen | |

The results for series HY/33 are expressed by the same symbols with a vertical line through them, a discontinuous line being used to join up the points. Total nitrogen is plotted as mg. per apple and as a percentage of the fresh

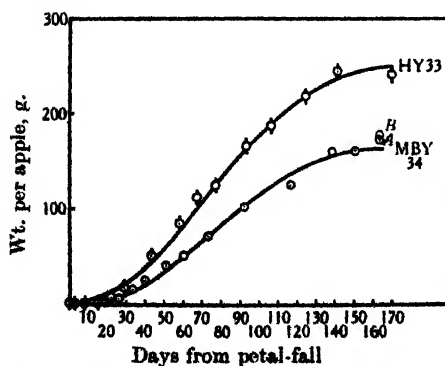


Fig. 1. Growth curves for the two series of apples.

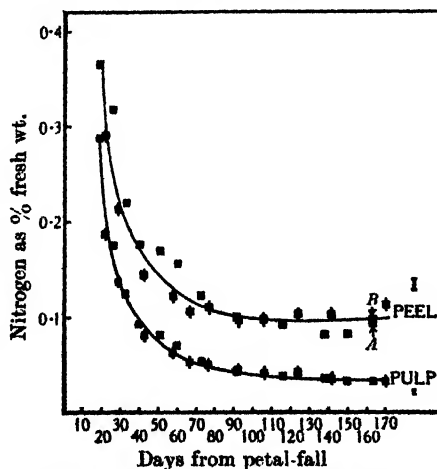


Fig. 2. Total nitrogen as % fresh weight.

A in the figures refers to results for fruits attached to the tree and B to results for fruits which had fallen into small bags attached to their branches at the time of the penultimate gatherings. The chemical results for the pulp were practically identical in both cases and are not plotted separately.

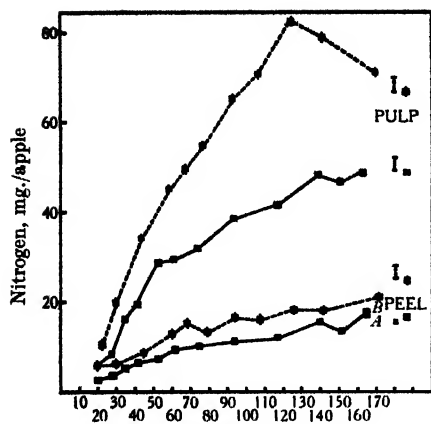


Fig. 3.

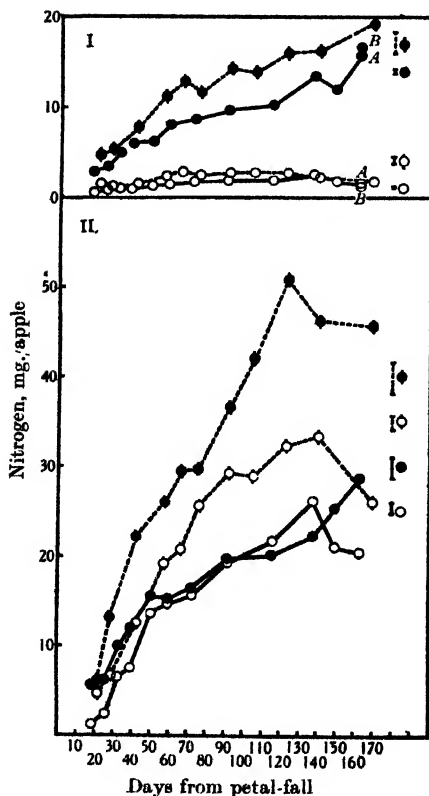


Fig. 4.

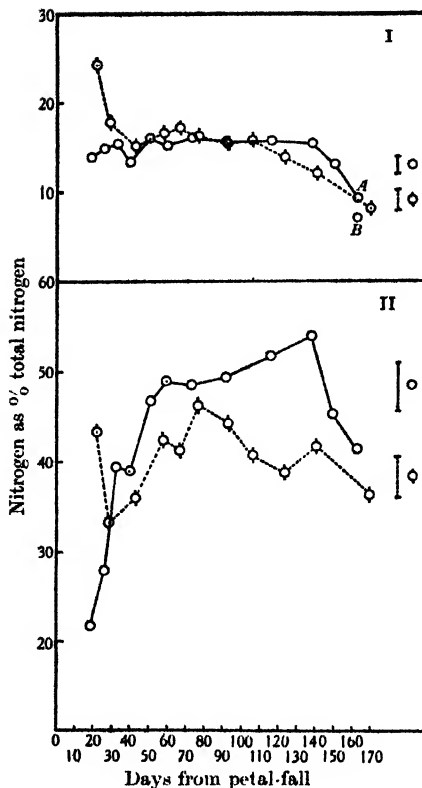


Fig. 5.

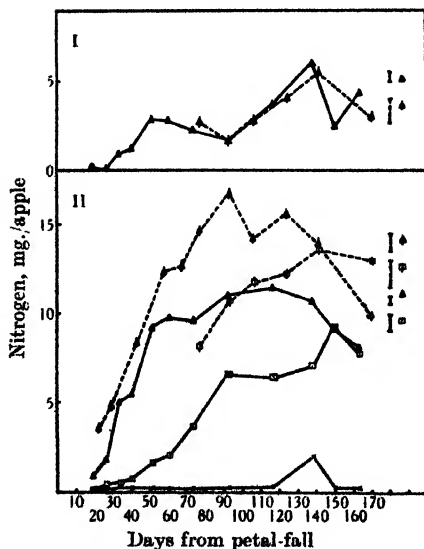


Fig. 6.

Fig. 3. Total nitrogen, mg./apple

Fig. 4. "Protein" total soluble nitrogen, mg./apple. I. Peel. II. Pulp.

Fig. 5. Total soluble nitrogen. I. Peel. II. Pulp.

Fig. 6. Pulp. Soluble N fractions, mg./apple. I. Peel. II. Pulp.

weight. The remaining nitrogen results are expressed in mg. per apple and as a percentage of the total nitrogen. Acid is expressed in g. per apple. Results for this fraction expressed as g. per 100 g. of water (the amount of water in the

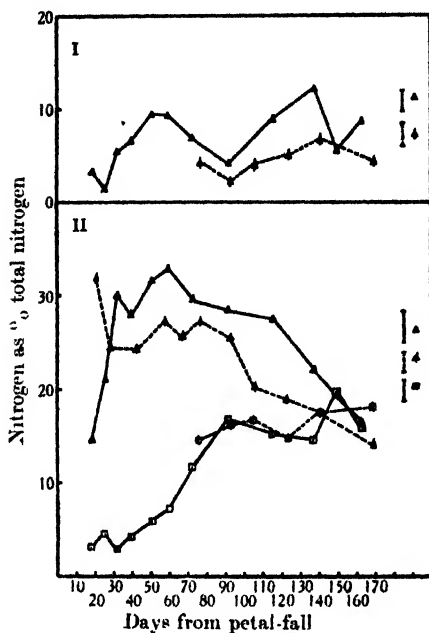


Fig. 7.

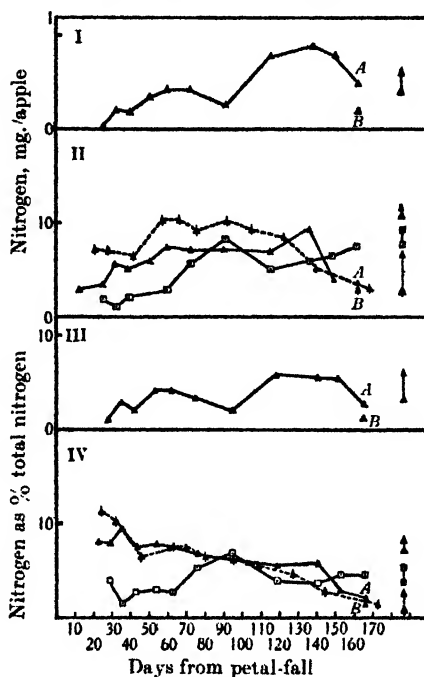


Fig. 8.

Fig. 7. Pulp. Soluble N fractions as % total nitrogen.

Fig. 8. Peel. Soluble N fractions. I and II in mg./apple. III and IV as % total nitrogen.

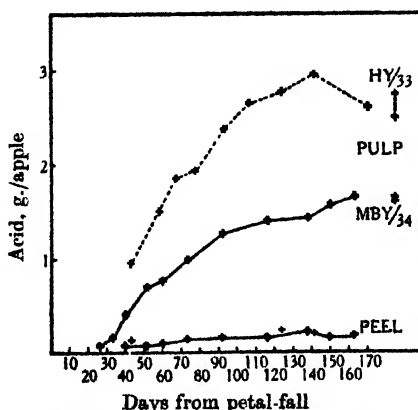


Fig. 9. Titratable acid (as malic).

tissue was obtained by evaporation at 55° *in vacuo* and determination of the weight of the dry residue) showed a steady fall throughout development in both series after about the 40th day.

The primary object of the present paper is to place on record the data expressed in the figures so that only the more obvious points arising from these data will be commented upon.

The different characters of the two sets of fruit are well illustrated in Fig. 1 from which it is seen that the maximum weight of the fruit in series HY/33 was approximately 245 g. and in series MBY/34 only about 165 g.

In the case of series HY/33 the amino-nitrogen was determined by a modification of the Van Slyke method giving only a partial correction for tannin (caseinogen precipitation method [Hulme, 1935, 1]). From estimations of the approximate tannin content of the fruit of this series at various stages during development [Hulme, 1935, 2] it was evident that from about the 80th day onwards, in the case of the pulp tissue, the amount of tannin present was insufficient seriously to affect the Van Slyke results obtained. Hence these latter results are given in the figures from gathering X onwards (see Table 1) and may be considered accurate enough for purposes of comparison with the general drift in series MBY/34. No amino-acid results for the peel of series HY/33 are given, since throughout the series the amount of tannin present was relatively large.

The most striking difference between the results for the two series is the opposite directions of the trend in total soluble and asparagine-nitrogen, when considered as a percentage of the total nitrogen (Figs. 5, II and 7, II), during the first period of development. Although this difference emphasises the need for a further study of this region, it may not be so significant as appears at first sight. At this early stage in the development of the fruits the cells of the tissue are densely filled [Tetley, 1931] and metabolic changes are relatively great. Furthermore, results for earlier samples of series HY/33, obtained for whole fruits (in these samples the apples were too small for peeling), gave, at day 8, total soluble nitrogen 24.5% of the total nitrogen and at day 15, 27.0%. If the results for this fraction at days 22 and 29 are calculated for the whole fruit the values obtained are 36.6 and 29.5% respectively. The asparagine-nitrogen figures for these four dates show a similar relation to one another. Hence it would appear that the soluble and asparagine-nitrogen results for day 22 (series HY/33) are abnormally high and further work may show that during this early stage of development the results for series MBY/34 more truly represent the general course of events.

From about the 30th day onwards there is a distinct similarity between the two series in the general drift of the nitrogen fractions when due weight is given to the values of the significant differences. This is especially striking in view of the different type of the fruit in each series. It would appear, therefore, that the processes underlying the recorded changes are fundamental in the development of apple fruits—at least of the Bramley's Seedling variety. Taking the results for the pulp as a whole three phases in the nitrogen cycle are apparent. An early stage of rapid increase in soluble nitrogen compounds, especially asparagine, is followed by a stage of relative equilibrium between protein and non-protein compounds. Finally when the fruit is mature a state of net synthesis of protein ensues. This last phase is striking and quite unexpected in a senescent organ. It is also a feature of mature apples when detached from the tree and will be considered more fully in later papers.

In the case of the peel, as might be expected, changes in the nitrogen fractions are small but, in general, run parallel with changes in the pulp. Again, the most noteworthy fact is the apparent synthesis of protein when the fruit has reached maturity.

Finally the present data may be considered in relation to the suggestions of Ruhland and Wetzel [1926; 1927] that acid formation in plants (other than members of the Crassulaceae [Wolf, 1931]) is intimately connected with nitrogen metabolism.

The low p_H of the sap of the Bramley's Seedling apple would place it in the class of plants called by Ruhland and Wetzel "ammonia" or "acid" plants, in which case ammonium salts should predominate over amides. Actually the reverse proves to be the case. From Fig. 6, II, it is clear that asparagine is greatly in excess of ammonium salts in the tissues of the apple fruit throughout the whole growth period examined. In fact only at one point is the latter fraction present in appreciable amounts. The ammonia-nitrogen results for series MBY/34 only are given since the results for series HY/33 are substantially identical with them with the exception that the maximum near the end of the growth period is considerably lower. Further, in considering Ruhland and Wetzel's contention that the plant acids arise chiefly in the course of protein metabolism the present data show that this is unlikely to be the case in the apple, for the amount of acid present (Fig. 9) is always greatly in excess of that of any of the nitrogen compounds. The evidence is not, however, entirely conclusive since both the amount of acid entering the fruit from the tree and the amount (if any) of the nitrogen compounds passing from the pulp tissue to the seeds are unknown.

SUMMARY.

1. Details are given of the methods of sampling and preparation of the fruit for analysis.
2. The separation of the total nitrogen into the two fractions protein- and non-protein-nitrogen is briefly discussed and reasons are given for the use of alcohol extraction to attain this end in the present instance.
3. Methods are given for the estimation of total nitrogen and of the various constituent fractions of the soluble nitrogen.
4. Graphs are presented showing changes in size of fruit, total nitrogen, total soluble nitrogen, ammonia-nitrogen, asparagine-nitrogen, amino-acid-nitrogen, "rest"-nitrogen and titratable acidity during the course of development, from petal fall to maturity, of fruit from two series of Bramley's Seedling apple trees. The nitrogen fractions are given for both peel and pulp of the fruits.
5. Certain points arising out of these results are briefly discussed and it is concluded that there are three stages in the nitrogen cycle of the developing apple fruit.
6. The results for free ammonia and for amino-acid-nitrogen and titratable acid provide evidence which appears to be inconsistent with the hypothesis of Ruhland and Wetzel that acid formation in plants is intimately connected with the nitrogen metabolism.

In conclusion the author wishes to acknowledge a Strathcona Research Studentship from St John's College, Cambridge, during the tenure of which the preliminary work here described was carried out. Grateful acknowledgment is also made to Prof. A. C. Chibnall for kind hospitality and advice on the determination of the nitrogenous constituents of plants received during a short stay in his laboratory.

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XLII. ON THE ACTION OF PARATHORMONE. II.

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It has been shown by numerous investigators that the administration of parathormone results in an immediate phosphate diuresis followed some hours later by a rise in the serum calcium. In a previous paper Goadby and Stacey [1934] showed that the phosphate diuresis was not accompanied by a rise in plasma inorganic phosphate and could not therefore be considered as the result of a liberation into the blood-stream of phosphorus held elsewhere in the body, and they brought forward evidence which suggested that parathormone produced this effect by direct action on the kidney.

The present work was undertaken to examine this point further, by observing whether or not parathormone produces a phosphate diuresis when the kidneys are severely damaged. Experiments similar in all respects to those previously recorded were carried out on patients in an advanced stage of renal insufficiency due to chronic nephritis.

Methods.

The patient was allowed a normal breakfast at 8 a.m., no lunch and a normal tea at 4 p.m. The experiment was conducted from 6 a.m. to 6 p.m., during which time half a pint of fluid—lemonade or water—was taken every 2 hours to ensure adequate urine excretion. The urine was collected during a preliminary period every 2 hours, *viz.* 6–8 a.m., 8–10 a.m., 10 to midday. At midday blood was taken from the antecubital vein and immediately afterwards 100 units of parathormone (Lilley) were injected intramuscularly. The urine was collected every hour after this up to 6 p.m. Blood was taken again at 2.30 p.m., which corresponded to about the maximum phosphate diuresis previously found in normals; a further sample was taken 24 hours after the injection. The following estimations were performed.

Plasma inorganic phosphate. Blood was drawn into a 10 ml. centrifuge-tube containing 2 drops of 20% potassium oxalate solution and the plasma separated at once. The method of Youngburg and Youngburg [1930] was used for this estimation.

Serum calcium. This was estimated by Clark and Collip's [1925] modification of the Kramer-Tisdall technique.

Urine inorganic phosphate. When albumin was present this was precipitated by the addition of an equal volume of 20% trichloroacetic acid to an aliquot portion of the urine. The estimation was done on the filtrate by the Youngburg technique.

Experiments were done on 3 cases of chronic nephritis with severe nitrogen retention, 1 case of congenital cystic kidneys with nitrogen retention, 1 case of acute nephritis during nitrogen retention and again during convalescence. Experiments were also done on 3 cases of renal disease without gross derangement of renal concentrating power, as shown by normal blood ureas and normal urea concentration tests, *viz.* 1 case of congenital cystic kidneys without

nitrogen retention, 1 case of acute nephritis during convalescence (*vide supra*), and 1 case of nephrosis. In none of the cases was there sufficient circulatory failure to prevent absorption of the parathormone.

DISCUSSION.

The experimental findings are given in the figure and tables and show clearly that when the renal function is grossly impaired parathormone does not produce a phosphate diuresis in any way comparable with that obtained in

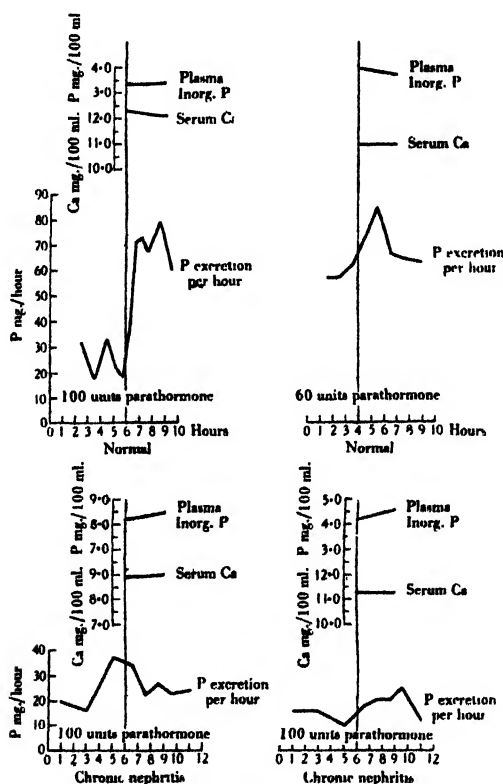


Fig. 1.

normal persons. Furthermore if the phosphate diuresis in normals were secondary to a rise in blood phosphate mobilised from the reserve stores, this rise would be very marked in those persons in whom the diuresis did not occur owing to renal damage. No such rise was observed, the small changes seen were insignificant compared with the amount of extra phosphorus excreted by normal persons after parathormone.

Further confirmation of our hypothesis is given by case 5, acute nephritis: during the stage of acute renal failure (blood urea 115 mg./100 ml.) the phosphate diuresis was small: after recovery, when the blood urea was normal, the phosphate diuresis was increased fourfold. (No such increase has been seen in normal persons when given parathormone injections at similar intervals of time.)

It will be seen that there is a rise in the serum calcium 24 hours after parathormone injection both in the normal and in the nephritic series: this suggests

Table I. *Response of normal and nephritic individuals to 100 units of parathormone.*

Case	Plasma phosphate variation in 6 hours + or - mg./100 ml.	Extra phosphate excretion in 6 hours mg.	Serum Ca rise in 24 hours mg./100 ml.
1. Chronic nephritis B.P. 210/130, B.U. 160	+0.4	27.3	1
2. Chronic nephritis B.P. 150/80, B.U. 150, U.C.T. 0.87	+0.4	32.9	2.4
3. Congenital cystic kidneys B.P. 185/105, B.U. 134, U.C.T. 1.2	+0.2	7.7	1.0
4. Arteriosclerotic kidneys B.P. 182/84, B.U. 170	+0.1	6.8	0.7
5. Acute nephritis B.P. 144/106, B.U. 115, U.C.T. 1.35	+0.1	76.0	1.0
5. Acute nephritis, recovered B.P. 116/70, B.U. 31, no albuminuria	-0.56	285.0	4.3
6. Congenital cystic kidneys B.P. 160/95, B.U. 115, no albuminuria	-0.15	166.0	1.2
7. Nephrosis. Plasma cholesterol 640 B.P. 124/72, albuminuria, casts + +	+0.02	325.0	1.8
8. Normal	-0.23	225.8	0.9
9. Normal	-0.59	415.4	2.0
		Extra phosphate excretion in 4 hours mg.	
10. Normal	-0.25	215.0	—
11. Normal	+0.13	138.0	—
12. Normal	+0.08	312.0	—
13. Normal	-0.36	135.6	—
14. Normal	-0.15	138.0	—

B.P. = Blood pressure in mm. Hg. B.U. = Blood urea in mg./100 ml. U.C.T. = Maximum % concentration of urine-urea in urea concentration test.

that the actions of parathormone in raising the blood calcium and in causing increased excretion of phosphorus are independent, for the Ca rises whether there is the initial large phosphate diuresis or not. These findings are in accordance with the observations of Ellsworth and Fitcher [1935], who showed that parathormone produces a rise in the blood calcium of nephrectomised dogs. Collip *et al.* [1934] also demonstrated that in nephrectomised animals the bones were depleted of calcium by injection of parathormone in the same way as in normal animals.

SUMMARY.

1. In persons in whom the excreting power of the kidneys is grossly impaired, parathormone fails to cause the same increased excretion of phosphorus in the urine as occurs in normal persons.

2. This was true in all 5 cases of renal damage, due to various causes, that have been examined.

3. In no case was there a large rise in the plasma inorganic phosphate after parathormone.

4. The hypothesis that parathormone acts directly on the kidneys to produce a phosphate diuresis is thus confirmed.

5. In the same 5 cases, the blood calcium rose in the 24 hours after the injection to the same degree as in normal persons.

6. It is therefore suggested that parathormone has at least two actions: one on the kidney, the other on the calcium stores of the body.

We wish to express our gratitude to the Physicians of St Thomas's Hospital for permission to use their cases.

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XLIII. THE AMINO-ACIDS OF COCKSFOOT PROTEIN WITH SPECIAL REFERENCE TO THE DICARBOXYLIC ACIDS AND PROLINE.

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IN continuation of the research dealing with the amino-acid composition of cocksfoot protein, of which the basic amino-acids have already been determined [Miller, 1935], an account is now given of the isolation of proline by the method of Kapfhammer and Eck [1927], combined with that of Spörer and Kapfhammer [1930], and of the isolation of aspartic and glutamic acids by two slightly different methods both based on the procedures of Foreman [1914], Dakin [1918; 1920] and Jones and his co-workers (*e.g.* Jones and Moeller [1928]). No direct evidence for the presence of either hydroxyproline or hydroxyglutamic acid was obtained. During the course of the work the monoaminomonocarboxylic acids have been obtained in crystalline form by the usual butyl alcohol method of Dakin; their mean N content has been determined but the separation of the individual amino-acids is as yet incomplete.

It has already been suggested and partly confirmed that the low N content of the grass proteins prepared in this laboratory is due to the presence of N-free impurity [Miller and Chibnall, 1932; Chibnall *et al.*, 1933; Miller, 1935]. The analysis of cocksfoot protein is now sufficiently far advanced to permit calculation of the amount of this adulterant, and in a discussion at the end of this paper it has been deduced that the grass proteins, if they could be prepared free from non-nitrogenous impurities, would contain about 16% N. This authenticates the assumption made by agricultural workers that the protein content of forage crops can be calculated from the "true protein" N by multiplying by the factor 6.25 (*i.e.* 100/16).

EXPERIMENTAL.

The dicarboxylic acids.

Exp. 1. 50 g. of a cocksfoot protein (AG), having a dry weight of 49 g. and N 13.2% of the dry weight, were hydrolysed with 270 ml. of 20% (1 : 1) HCl, by heating on a steam-bath for 1–2 hours and then boiling under reflux for 36 hours in an oil-bath. 5.34 g. of "acid humin", containing 3.80% N, were separated. The clarified solution contained 6.16 g. N, making a total, with the humin-N, of 6.36 g. The latter figure will be referred to as "protein-N", on which all yields, in terms of N, will be calculated. The solution was concentrated to about 300 ml. (this and all subsequent distillations were carried out *in vacuo* below 50°) and treated with solid barium hydroxide until alkaline to litmus. The "alkaline humin" was filtered off, washed with hot water and dried at 105°. This weighed 5.10 g., and contained 2.23% N. The total humin (acid + alkaline) weighed 10.44 g. and contained only 0.345 g. N; it represents therefore the major part of the non-protein impurity present in the protein preparation and by

difference the protein itself must have weighed 38.56 g. and have contained between 6.015 and 6.36 g. N, i.e. between 15.6 and 16.4 %. This point is developed in the discussion.

The amino-acid filtrate was concentrated to about 400 ml. and a Foreman precipitation carried out, but using barium hydroxide in place of calcium hydroxide since the barium ion was easier to remove. In this and all subsequent Foreman precipitations the procedure was as follows, with the exception of the volume of alcohol added and of certain other variations which will be stated in each case. The solution was saturated with barium hydroxide at room temperature by adding the solid material with shaking till a little remained undissolved and was then poured with stirring into 93 % alcohol (in this case, 5 vols.) and the mixture left at room temperature for 2 days. The insoluble barium salts were filtered off, washed once with alcohol and suspended in water, and the barium was removed exactly with H_2SO_4 . In this instance 2 l. of water were used in an attempt to dissolve the barium salts completely, but a small amount of an insoluble substance, containing 0.037 g. N, still remained. Later experience suggested that considerably less water would have effected the same result. The filtered solution, containing 1.63 g. N, was treated with 2.5 l. of alcohol and left at 0° overnight. The barium salts were decomposed as before to give a solution (solution A) containing 0.705 g. N. The alcoholic filtrate from this operation was evaporated at 40° to dryness to remove alcohol. The residue was practically all dissolved in 400 ml. of water, the solution treated with 500 ml. of alcohol, and left in the ice-chest overnight. The precipitated barium salts were decomposed as before; the resulting solution (solution B) contained 0.181 g. N, of which 87.2 % was amino-N. (Solutions A and B were worked up for glutamic acid as described later.)

This last alcoholic filtrate, after removal of solid material (1.9 g. probably largely barium carbonate with some tyrosine) which had been deposited while the solution had been standing, was concentrated at 40° to remove alcohol, and barium removed with H_2SO_4 . The solution contained 0.672 g. N (96 % amino-N), and 6.19 g. solids. It was concentrated to 100 ml. and a Foreman precipitation carried out, using $1\frac{1}{2}$ vols. of alcohol and leaving at 0° overnight. The barium precipitate was worked up in the usual way; the resulting solution (solution C) contained 0.092 g. N (all amino-N). The alcoholic filtrate was treated as before and another Foreman precipitate taken out from 50 ml. of solution, using an equal volume of alcohol; the solution (solution D) derived from the barium salts contained 0.0511 g. N.

Glutamic acid. Solutions A and B were combined, freed exactly from SO_4^{--} , and concentrated at 40° to 80 ml. After saturation at 0° with dry HCl, the solution was left in a glass-stoppered bottle for a week at 0°. The mass of crystals was collected on a sintered glass Gooch crucible, washed with a little cold concentrated HCl and dried in a vacuum desiccator over NaOH sticks: yield 1.13 g. The filtrate was concentrated to 25 ml., resaturated with HCl and a second crop (1.92 g.) obtained. The mother-liquor was further concentrated to 15 ml., resaturated as before and a third crop (2.73 g.) procured. At this stage solutions C and D were added to the mother-liquor, leading to a fourth crop (0.51 g.). The total yield of glutamic acid hydrochloride, allowing for aliquots withdrawn for analyses, was 6.73 g., or 5.39 g. of the free amino-acid. This represents a yield of 13.1 % of the original weight of protein (recalculated on a basis of 16 % N); or, in terms of N, 8.03 % of the protein-N. The recrystallised product contained 7.68 % N; calculated for glutamic acid hydrochloride, 7.61 %. The ash was negligible.

The isolation of aspartic acid in this experiment will not be described in detail since the yield was low, probably owing to certain unnecessary steps in the procedure, the acid being precipitated successively as its barium salt (Foreman precipitation), as its lead salt [Dakin, 1918] and finally isolated as the copper salt.

Exp. 2. 100 g. of cocksfoot protein (AB), having a dry weight of 94.4 g. and N 13.7 % of the dry weight, were hydrolysed as in the previous experiment. 11.24 g. of humin, containing 3.93 % N, were obtained. The solution contained 12.26 g. N, or with the humin-N the total would be 12.70 g. ("protein-N"). The solution was freed from Cl^- with silver sulphate, and the excess Ag^+ removed by means of H_2S . The excess of the latter was removed by concentration; the solution then contained 11.91 g. N, a loss of 0.35 g. N. H_2SO_4 was quantitatively removed with barium hydroxide solution, resulting in a solution reacting just acid to litmus. The barium sulphate was centrifuged off and digested three times successively with water on a boiling water-bath. The solution and washings were concentrated at 45° to about 700 ml. (if the volume was reduced lower than this a solid crystalline mass resulted) and left overnight. The cream coloured solid was filtered off, washed and dried at 105° . It weighed 3.25 g., contained 7.39 % N, and apparently consisted chiefly of tyrosine and leucine. In Table I, given later, this product is referred to as monoamino-acid mixture A.

The filtrate, containing 11.40 g. N, was concentrated to 350 ml., divided equally into four 500 ml. centrifuge bottles stoppered with rubber bungs and shaken for 10 min. in a shaking-machine with 1200 ml. of *n*-butyl alcohol. The liquids were then centrifuged and the clear upper layer carefully syphoned off and concentrated at 40° under reduced pressure. The aqueous phase remaining was further extracted with butyl alcohol as before. After this second extraction the aqueous solution was made neutral to litmus with barium hydroxide solution. (This was an improper step as the glutamic acid hydrochloride subsequently isolated contained barium chloride.) The solution was then concentrated to about 300 ml. (75 ml. in each bottle) and given 8 further extractions, using 5 vols. of butyl alcohol. The aqueous phase, now containing 6.87 g. N (54.1 % of the protein-N), was concentrated to 200 ml. (50 ml. in each bottle) and further extracted 5 times with 8 vols. of butyl alcohol.

The butyl alcohol extracts were concentrated to about 500 ml. and the cream coloured amino-acids filtered off and washed with butyl alcohol followed by acetone. They weighed 38.0 g. and contained 11.9 % N. This material is referred to in Table I as monoamino-acid mixture B. The butyl alcohol filtrate contained 0.525 g. N consisting largely of proline-N.

Glutamic acid. The aqueous phase, containing 5.91 g. N. (46.5 % of the protein-N), was concentrated to about 130 ml., saturated with dry HCl at 0° and glutamic acid hydrochloride isolated in the usual way: 10.2 g. (in 3 crops) were obtained. The mother-liquor was repeatedly evaporated to a syrup from water to remove free HCl, taken up in water and made slightly alkaline to litmus with solid barium hydroxide. The small amount of humin was removed by filtration, the filtrate concentrated to 100 ml. and a Foreman precipitation carried out using 10 vols. of alcohol and leaving overnight. The alcohol filtrate (1), containing 2.09 g. N, was treated later. The barium salts were worked up as usual; the resulting solution, containing 2.605 g. N (equivalent to 20.5 % of the protein-N), was concentrated to a thin syrup and saturated with HCl at -10° : 2.74 g. of glutamic acid hydrochloride were obtained. In this experiment then a total of 12.94 g. had been collected. It contained 13.5 % of ash (for the reason stated previously) and 3.5 % of ammonia-N; the N-content of the ash- and ammonia-

Table I.

	Yield				
	Expressed as N in % of protein-N	As % of total wt. of protein (N, 16.0 %)	Actual wt. isolated g.	N in amino- acid %	*
Arginine	13.6	6.11	5.62	32.2	2.520
Histidine	1.66	0.89	0.82	27.1	0.309
Lysine	5.27	3.98	3.67	19.7	1.007
Proline	2.0	2.52	2.21	12.17	0.374
Glutamic acid	8.03	13.10	10.12	9.5	1.340
Aspartic acid	3.25	5.32	4.30	10.5	0.629
Monoamino-acid mixture A	1.9	4.04	3.26	7.42	0.337
Monoamino-acid mixture B	35.6	47.0	38.0	11.9	6.290
Monoamino-acid mixtures C and D	3.29	3.64	2.94	14.3	0.585
Ammonia	6.0	1.15	0.93	82.3	1.069
(Humin)	5.4	—	—	—	—
Total	86.00	87.75	71.87	—	14.460

* % of N contributed by each amino-acid (or mixture): e.g. for arginine, $\frac{5.62 \times 32.2}{71.87} = 2.520$.

free hydrochloride was 7.85 %. Calculated from this N content the yield of free glutamic acid was 11.5 % of the original weight of protein (N, 16.0 %), or in terms of N, 7.05 % of the protein-N.

Aspartic acid. The mother-liquor from the above hydrochloride, after removal of free HCl by concentration, contained 2.35 g. N (87.2 % amino-N). It was concentrated at 40° to about 100 ml. and another Foreman precipitation carried out, using 1½ vols. of alcohol and leaving 1 hour at 0°. The alcoholic filtrate (2), containing 1.175 g. N, was treated later. The barium salts gave a solution containing 1.055 g. N. The solution (volume 250 ml.) was heated to boiling and about 10 g. of Kahlbaum's cupric hydroxide added; the excess was filtered off, washed with hot water, and the filtrate and washings were left overnight at 2°. The copper aspartate was filtered, washed with a little cold water and dried at 105° overnight: weight, 6.28 g. This is a yield of free aspartic acid of 5.32 % of the original weight of protein (N, 16.0 %); or expressed as aspartic acid-N, 3.25 % of the protein-N. Analyses were done on the air-dry copper aspartate (containing 4½ H₂O), since it was found difficult to render the salt anhydrous. After one recrystallisation the following values were obtained: N, 5.13; Cu, 22.4 %. C₄H₅O₄NCu, 4½ H₂O requires N, 5.23; Cu, 23.0 %.

The filtrate on concentration yielded no further crop of copper aspartate; it contained 0.547 g. N, of which 85.2 % was amino-N. Since the N in the solution was not all in the amino-form, it was thought that basic amino-acids might be present. The solution (250 ml.) was therefore treated with 18 ml. of concentrated HCl followed by 20 % phosphotungstic acid in 3 % HCl solution until precipitation was complete. The precipitate was filtered off, washed with the usual reagent, and the filtrate and washings were freed from phosphotungstic acid with the usual 1 : 1 amyl alcohol: ether mixture. The aqueous solution was evaporated to dryness under reduced pressure and the residual syrup dissolved in water. The solution contained 0.446 g. N (all amino-N). No further amounts of glutamic and aspartic acids could be separated by the usual methods and an attempt to isolate hydroxyglutamic acid by Dakin's procedure [1918] was unsuccessful.

Monoamino-acid mixtures. The alcoholic filtrates (1) and (2) mentioned above were combined, concentrated at 40° to remove ammonia and alcohol, taken up

in water, and freed from barium with a slight excess of H_2SO_4 . The resulting solution, containing 3.27 g. N, was treated, in a volume of 1.5 l., with 20 % phosphotungstic acid in 5 % H_2SO_4 to complete precipitation, in order to remove bases. The mixture was left overnight, centrifuged and the precipitate washed with 3 % phosphotungstic acid (in 5 % H_2SO_4). The supernatant solution and washings, containing 1.073 g. N, were freed from phosphotungstic acid by making alkaline with hot barium hydroxide solution, filtering off the precipitate and washing with alkaline water. Barium was removed from the filtrate, which then contained 0.880 g. N (86.5 % amino-N). All Cl^- was next removed with silver oxide and the excess Ag^+ with H_2S . After concentration the solution then contained 0.841 g. N. It was concentrated to 50 ml. and extracted 10 times with 4 vols. of butyl alcohol. The solid monoamino-acids obtained from the extract weighed 1.94 g. and contained 14.3 % N. In Table I, this material is referred to as monoamino-acid mixture C.

The aqueous phase, containing 0.378 g. N, gave no Foreman precipitate on a small sample; it was therefore further treated with 20 % phosphotungstic acid in 5 % H_2SO_4 solution and the copious precipitate removed on the centrifuge and washed twice with the usual reagent. The clear solution and washings, containing 0.298 g. N, were freed from phosphotungstic acid with barium hydroxide solution and then exactly from barium with H_2SO_4 . The solution (0.253 g. N) was then concentrated to 8 ml., 3 vols. of alcohol added and the turbid solution left for some days in a refrigerator. The white solid was filtered off and dried in a vacuum desiccator over phosphorus pentoxide: yield 0.996 g., containing 14.22 % N, and therefore probably representing approximately the same mixture of amino-acids (including glycine, alanine and possibly serine) as mixture C. This is referred to in Table I as monoamino-acid mixture D.

Proline.

To obtain some idea of the maximum quantity of proline present in cocksfoot protein the following experiment was performed.

Exp. 3. The same protein (AB) was used as in the previous experiment. 2.078 g. were hydrolysed by boiling with 35 ml. of 20 % HCl for 22 hours. The humin contained 0.010 g. N and the filtrate 0.255 g., so that the protein-N was 0.265 g. The filtrate was made alkaline with a slight excess of an aqueous suspension of magnesium oxide and ammonia and humin removed in the usual way. The solution then contained 0.239 g. N, of which 0.201 g. was amino-N (including the $\epsilon\text{-NH}_2$ group of lysine). Thus the total non-amino-N was 0.038 g., or 14.34 % of the protein-N; this will include the non-amino-N of proline and hydroxyproline, arginine, histidine and possibly some diketopiperazine. Now this particular cocksfoot protein had not been analysed for the basic amino-acids, but assuming it to contain the same percentage as the cocksfoot protein used in the large scale analysis described previously [Miller, 1935], the non-amino-N due to arginine and histidine would be 11.31 % of the protein-N. Thus the maximum amount of proline-N and hydroxyproline-N (including any diketopiperazine-N) would be 3.03 % of the protein-N.

For the quantitative isolation of proline the following method [Kapfhammer and Eck, 1927; Spörer and Kapfhammer, 1930] was adopted.

Exp. 4. For convenience the actual solution from which the basic amino-acids had already been removed by the Vickery large scale analysis [Miller, 1935] was used, although it should be realised that the latter involved losses which might conceivably have included small amounts of proline and hydroxy-

proline. The filtrate from the lysine phosphotungstate was freed from phosphotungstic acid by means of barium hydroxide and then from excess of the latter by the addition of H_2SO_4 to give a reaction slightly acid to Congo paper. The final solution was concentrated at 45° to 150 ml., when solid began to separate. The mixture was cooled in ice to freeze out as much of the material as possible, and filtered. The dried precipitate weighed 8.42 g. On concentration of the filtrate 2.88 g. more were obtained, making 11.3 g. in all. The combined precipitates contained 10.0% N and probably therefore consisted largely of leucine (N, 10.69%). The filtrate was concentrated to 120 ml. and treated at 60° with an 8% solution, also at 60° , of 30 g. of ammonium reineckate (recrystallised from water at 60°). On cooling crystallisation began and was allowed to proceed to completion in the refrigerator overnight. The precipitate was filtered off, washed with a little ice-cold water and dried over phosphorus pentoxide *in vacuo*: yield 23.5 g. On concentration of the filtrate and addition of more ammonium reineckate solution 11.5 g. more were obtained, making a total of 35 g. The latter was suspended in 300 ml. of 50% methyl alcohol, warmed, and a hot solution of 60 g. of copper sulphate (A.R.) added with stirring. The brown precipitate was filtered off, washed, and the filtrate saturated with sulphur dioxide and left overnight. The precipitate was filtered off, washed, and the filtrate concentrated to small bulk. To this hot solution was added silver sulphate to remove the remainder of the CNS^- , and then after cooling, H_2S was passed to remove Cu and Ag. The resulting solution was concentrated to 300 ml., 50 g. of barium hydroxide added and all ammonia distilled off *in vacuo*. The precipitate was centrifuged off, washed and the liquid freed from barium with H_2SO_4 . The solution then contained 0.556 g. N, of which 0.232 g. was amino-N; i.e. 0.324 g. of non-amino-N, or 2.76% of the protein-N. The solution was evaporated to dryness at 45° , and the solid residue triturated with hot alcohol and left overnight. The precipitate was filtered off, washed with absolute alcohol and dried over phosphorus pentoxide in a vacuum: weight 1.243 g. It contained 10.0% of N, all of which was amino-N, and therefore did not contain any hydroxyproline, which would normally appear at this stage if present in appreciable amount in the original protein.

The filtrate was evaporated to a syrup which was dissolved in about 90 ml. of 50% alcohol and treated with 100 ml. of a 5% solution of cadmium chloride in alcohol. The solution was left in the ice-chest overnight; the crystals which had then separated were filtered off and the filtrate concentrated to 50 ml. More cadmium chloride solution (50 ml.) was added and the liquid left in the ice-chest over a week-end. A second crop was isolated giving a total of 5.06 g. (Found: N, 4.65. $\text{C}_5\text{H}_9\text{O}_2\text{N} \cdot \text{CdCl}_2$ requires N, 4.71%.) Expressed as weight of proline this represents a yield of 2.52% of the original weight of protein (N, 16.0%); or in terms of proline-N, 2.01% of the protein-N. An attempt was made to improve this yield by adopting Bergmann's method [1935], but pure proline rhodanilate could not be isolated, probably owing to the large amount of humin and relatively small proportion of proline present in the grass proteins as compared with gelatin.

DISCUSSION.

The results of the foregoing experiments in which the amino-acids (or mixtures of them) have been actually isolated, are given in Table I, along with the basic amino-acids [Miller, 1935] and ammonia. Reading from left to right, the first two columns of figures give the yields of the various amino-acids; the last three columns are entered in order to arrive at the total mean percentage of

N in the sum of all the isolated amino-acids (or mixtures), viz. 14.46 %. (Humin with its low N-content has been purposely excluded from this calculation because the N-free impurity present in the original protein is concentrated in this fraction.) Now when a protein is completely hydrolysed, a water molecule is added at every peptide (and amide) linkage forming an α -amino-group (and ammonia). Of the above 14.46 %, 11.99 % is α -amino-N (including ammonia-N); i.e. $\frac{18 \times 11.99}{14} = 15.4$ % of water molecules have been added on to the original weight of protein in the formation of the amino-acids. Thus when this water is allowed for, the N-content of the protein, if non-nitrogenous adulterants be excluded, would be 17.1 %. Of course this value is calculated on a total yield of amino-acids of 86.0 %. The remaining 14 % of amino-acids would almost certainly contain a lower percentage of N than this, but even assuming an average figure of 10 %, the calculated N content in the pure cocksfoot protein (and experience suggests the proteins of most other common grasses also) would still be 16.0 %. This value is in agreement with that calculated previously on the humin-free protein (AG). Accordingly for purposes of comparison with the N contents of other practically pure plant proteins such as edestin (18.6 %), zein (16.1 %), proteins from the leaves of spinach (16.25 %) and alfalfa (15.7 %), a general figure of 16 % N for pure grass proteins can be safely assumed and this value has been adopted throughout this paper in calculating the yields of amino-acids in percentages of the total weight of protein.

The total N recovered in the amino-acids (although in some cases as mixtures not yet separated) so far isolated from the grass proteins, viz. 86.0 %, compares favourably with the corresponding figures for edestin, 84.4 %; and caseinogen, 82.3 %.

SUMMARY.

Glutamic acid, aspartic acid and proline have been isolated from cocksfoot protein in the following respective yields: 13.10, 5.32 and 2.52 % of the protein; or in terms of N, 8.03, 3.25 and 2.0 % of the protein-N. The presence of hydroxyglutamic acid and hydroxyproline could not be demonstrated.

Evidence is adduced that the protein, if it could be prepared pure—as yet impracticable—would contain about 16 % N.

The author desires to thank Prof. A. C. Chibnall for much help and advice, and Imperial Chemical Industries for a grant covering the cost of this research.

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XLIV. CYSTINE AND THE DIETARY PRODUCTION OF FATTY LIVERS.

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(Received December 30th, 1935.)

In a previous paper it was shown that the amount of fat appearing in the livers of rats receiving a diet of low choline content (choline intake 1.5 mg. per rat per day) and containing 40 % of fat was controlled by the amount of caseinogen present in the diet. In a later paper the findings concerning this lipotropic action of caseinogen were extended to embrace the glyceride fraction of the "cholesterol" fatty liver [Channon and Wilkinson, 1935; Beeston *et al.* [1935], whilst more recently results were reported which suggested that the caseinogen used in our diets possessed a lipotropic action equivalent to 8 mg. of choline per g. [Beeston and Channon, 1935].

In the first-mentioned paper it was suggested that further information concerning this control of liver fat by dietary protein fractions might be obtained by supplementing diets low in caseinogen with individual amino-acids, and a systematic study of this problem is in progress. The present paper records results already obtained with cystine. Contrary to the original expectation, it has been found that supplementing a diet low in choline and containing 5 % caseinogen and 40 % fat with small amounts of cystine causes a profound increase in the amount of liver fat.

EXPERIMENTAL.

The control groups of animals, 8-10 in each group, received a diet consisting of caseinogen 5, beef dripping 40, marmite 5, glucose 44, salt mixture 5 and cod-liver oil 1 part. Other groups received this diet supplemented with amounts of cystine varying from 0.05 to 1.0 % of the dry weight of the diet. The cystine was thoroughly incorporated with the protein before admixture with the rest of the diet and the amount ingested calculated from the food intake which was measured daily. The periods of the experiments varied from 14 to 21 days. At the end of each experiment the animals were killed by guillotine and determinations were made of the amount of unsaponifiable matter and fatty acids present in the livers by the methods previously used [Channon and Wilkinson, 1935], and in the tables which follow the "fat" content of the liver represents the sum of the fatty acids and the unsaponifiable matter.

In all the experiments save No. 1, in which the livers were pooled, determinations of the fat contents of the individual livers were made; for brevity however the average results obtained for the different groups in the various experiments will form the basis of discussion and the individual figures for one experiment only will be discussed in detail.

In Table I there are recorded the essential practical data concerning all the experiments. The figures show that the food intake of the animals in the different experiments was adequate and the amounts eaten by the control and experimental groups were quite comparable; they show further that any changes in body weight during the experiments were too insignificant to play any part in the interpretation of the results. Because the variations in the final weights

Table I. *Experimental data and analyses of livers of rats receiving basal diet with and without added cystine. (Mean values.)*

Exp.	No. of animals	Cystine content of diet %	Food intake g./rat/day	Av. final body wt. g.	Av. change in body wt. \pm % initial body wt.	Period of exp. days	Av. wt. of fat in liver g.	Av. wt. of liver g.
1. Control	9	—	10.0	197	- 2.5	20	0.87	7.07
Control + cystine	10	1	8.0	204	- 0.1	—	2.13	10.20
2. Control	9	—	8.3	168	+ 0.2	21	2.59	8.43
Control + cystine	10	0.2	8.7	183	+ 17.3	—	5.04	12.73
Control + cystine	10	0.6	7.8	170	+ 4.0	—	3.79	10.28
3. Control	9	—	6.9	145	- 4.9	14	1.16	6.98
Control + cystine	8	0.2	6.9	146	- 4.0	—	2.24	9.20
4. Control	10	—	7.3	141	- 6.5	18	1.70	7.16
Control	8	—	5.7	139	- 8.1	—	1.71	7.27
Control + cystine	10	0.05	6.5	143	- 4.4	-	2.04	7.66
Control + cystine	8	0.1	7.5	155	- 1.7	-	3.09	9.27
Control + cystine	10	0.2	6.7	149	- 3.2	—	2.10	8.15
Control + cystine	10	0.4	6.7	153	- 2.9	-	3.04	9.42
Control + cystine	10	0.6	7.3	156	\pm 0.0	—	4.04	10.76

attained by the animals of the different groups prevent ready comparison of the individual figures recorded in Table I, the more essential figures are presented in Table II, calculated from the final body weights on the basis of the 100 g. rat, together with the daily cystine intake per animal. The figures in Table II show

Table II. *Fat in liver of animals receiving basal diet with and without added cystine. (The figures refer to the 100 g. rat.)*

Exp.	No. of animals	Liver as % of body wt.	Fat in liver % fresh wt.	Wt. of fat in liver g.
1. Control	9	3.60	23.74	0.745
Control + cystine (80 mg.)	10	5.26	39.65	1.921
2. Control	9	5.12	26.34	1.365
Control + cystine (17.4 mg.)	10	7.10	37.38	2.845
Control + cystine (46.8 mg.)	10	6.07	36.34	2.249
3. Control	9	4.83	14.54	0.799
Control + cystine (13.8 mg.)	8	6.16	24.71	1.342
4. Control	10	5.20	21.67	1.214
Control	8	5.16	21.18	1.263
Control + cystine (3.25 mg.)	10	5.38	24.03	1.644
Control + cystine (7.50 mg.)	8	6.41	34.50	2.158
Control + cystine (13.40 mg.)	10	5.56	24.40	1.409
Control + cystine (26.80 mg.)	10	6.10	27.86	1.981
Control + cystine (43.80 mg.)	10	6.59	35.42	2.593
Mean control	Total 45	4.78	21.50	1.077
Mean control + cystine	Total 86	6.06	31.78	2.02

the profound effect of cystine in increasing the fat content of the livers of animals receiving this particular diet. Thus in Exp. 1 the weight of fat in the liver is some $2\frac{1}{2}$ times as great as in those of the control animals, a result which is compounded from two factors, namely the increase in the percentage of fat in the liver from 23.74 to 39.65 % of the fresh liver weight, together with the increase in the size of the liver from 3.60 to 5.26 % of the body weight.

The results for Exp. 2 in which the cystine intakes of the two cystine groups were reduced to 17.4 and 46.8 mg. respectively are scarcely less striking. Here

again the amount of liver fat is more than doubled in the one case and some 60 % greater in the other, whilst the percentages of fat in the liver are 37.38 and 36.34 % of the wet weight, compared with the 26.34 % of the control animals. There are also similar corresponding increases in the liver size.

Exp. 3 was then carried out on groups of animals introduced from an outside source and these received 13.8 mg. of cystine daily. These animals, which were utilised as soon as they were imported into the laboratory, were nervous and unaccustomed to handling, a fact which may perhaps account for the low fat percentage (14.54 %) given by the control group. The same results appear however, for the liver fat is increased to almost double the control value, with increases in the percentage of fat in the liver and the liver size of the same order as in the previous experiments.

In an endeavour to determine whether there was any relationship between the amount of cystine ingested and the amount of liver fat which resulted, Exp. 4 was carried out. Because of large individual variations it was deemed wise to run two control groups of animals in this experiment, whilst five groups received added cystine. From the figures in Table II it does not appear that there is a quantitative relationship between the amount of cystine ingested and the weight of fat in the liver, for there is no regular gradation with the increasing cystine intake, whilst one group, *i.e.* those receiving 13.4 mg., has given a figure for liver fat which is no more than 16 % greater than the controls. It appears possible that this lack of gradation with increasing dosage may be due to the relatively small groups of animals used, because of the large individual variation which occurs, particularly among cystine-fed animals. This point will be discussed in the next section. The results of Exp. 4 however bear out those of the first three experiments.

It seemed worth while to draw up mean values for all the control animals and all those which received cystine, irrespective of the amounts of cystine received and the duration of the experiments. These figures appear at the bottom of Table II, where the mean value for the weight of liver fat for 45 control animals is 1.077 g., whereas that for 86 animals which received cystine is 2.02 g. This 87 % increase is due to two factors, the increase in liver size from 4.78 to 6.06 % of the body weight and the increase of the liver fat percentage from 21.50 to 31.78 % of the fresh weight. It appears then from these results that the effect of the inclusion of small amounts of cystine in a diet which contains 40 % of fat and is low in choline is to double the amount of the already high deposition of fat resulting from the control diet. It is to be remembered that the liver of the normal 100 g. rat weighs about 3.5 g. and contains about 0.12 g. of total fatty acids.

In order to make clear to other workers the great variations which may occur, the individual values obtained for the animals in a typical experiment, Exp. 2, are recorded in Table III, in which the figures again refer to the 100 g. rat.

In the control group the liver varies from 3.98 to 6.69 % of the body weight, whilst in the two cystine groups the variations are from 3.53 to 11.88 % and 4.91 to 7.96 % respectively. The overlap shown in these figures appears also in the percentage of fat in the liver. Thus in the control animals the variations are from 14.33 to 40.35 %, whilst in the cystine groups they are from 15.18 to 48.96 % and from 29.58 to 43.14 %. Hence a similar overlap occurs in the actual weight of fat in the liver. In the control group this varies from 0.657 to 2.271 g., whilst in the cystine groups it is from 0.536 to 5.817 g. and 1.371 to 3.434 g. respectively. Superficially it might appear that these individual variations are such as to render dubious the finding that the addition of cystine

Table III. *Individual values for liver fat of animals receiving basal diet with and without added cystine (Exp. 2).*

Control				Control + 0.2% cystine				Control + 0.6% cystine			
Animal no.	Liver wt. g.	Liver fat % fresh wt.	Fat in liver g.	Animal no.	Liver wt. g.	Liver fat % fresh wt.	Fat in liver g.	Animal no.	Liver wt. g.	Liver fat % fresh wt.	Fat in liver g.
1	5.09	30.35	1.545	10	5.59	31.52	1.762	20	7.24	35.85	2.595
2	5.98	37.96	2.271	11	5.71	37.83	2.160	21	4.91	29.58	1.452
3	4.47	24.23	1.082	12	8.34	42.41	3.535	22	7.96	43.14	3.434
4	3.98	16.50	0.657	13	6.78	40.13	2.720	23	6.37	39.64	2.524
5	6.69	40.35	2.179	14	6.63	39.00	2.586	24	6.67	41.38	2.760
6	5.55	14.33	0.795	15	9.13	48.53	4.431	25	5.58	32.74	1.827
7	4.94	18.82	0.930	16	5.51	27.46	1.513	26	4.60	29.78	1.371
8	5.53	19.14	1.058	17	3.53	15.18	0.536	27	5.28	38.58	2.036
9	4.97	35.34	1.757	18	7.92	42.85	3.394				
				19	11.88	48.96	5.817				
Mean	5.12	26.34	1.365		7.10	37.38	2.845		6.07	36.34	2.249

has had an effect, but statistical analysis shows that the results recorded in Table III are significant. Further, the similar results of Exps. 1, 3 and 4 seem to provide ample confirmation of the finding. Necessity for brevity precludes discussion in detail of the distribution of the figures about the so-called mean value and this distribution must be seen by inspection of Table III.

Typical difficulties of individual variations encountered may be illustrated by reference to these animals. The liver of rat No. 17 (0.2% cystine) was but 3.53% of the body weight (which is the usual figure for the normal animal), and contained 15.18% fat; there was thus only 0.536 g. present in this liver. Yet the liver of animal No. 16, which had the next smallest liver of this group, constituted 5.51% of the body weight, contained 27.46% fat and there were present in it 1.513 g. of fat, a quantity nearly three times as great. Further, the liver of rat No. 19 contained 5.817 g. fat, which is eleven times as much fat as that of No. 17. The considerable variations in the amount of fat in the livers of rats receiving choline-free diets has been discussed by Best and Channon [1935], but has no explanation at present. It does not appear to be due to varied food intake, nor can these individual variations in this type of experiment be overcome by use of litter-mates [Channon and Smith, 1936]. The addition of cystine both in the experiment under discussion and in the others described in this paper appears to make for even greater individual variations.

It is of some importance to draw attention to the magnitude of the changes of the livers of some of the cystine-fed animals. The most striking example is that of rat No. 19, the liver of which was 11.88% of the body weight and which contained 48.96% of its wet weight as fat, or 5.817 g. The presence of 5.817 g. of fat in the liver of a 100 g. animal is very striking, for it has to be remembered that the normal animal contains some 0.12 g. of total fatty acid in its liver. Further, about 90 mg. of this fatty acid are present as phosphatide, and therefore the amount of the glyceride present in the liver of this particular rat is something like 200 times greater than the normal. Further, this weight, 5.817 g., is approximately $1\frac{1}{2}$ times the fresh weight of the liver of the normal animal! Although we did not estimate the amount of body fat in these animals, it is usual to find a figure of something like 12% after experiments in which rats have received the control diet employed. This means then that the liver of this particular animal contained an amount of fat equal to one-half of the total fat in the body of the normal control animal.

It seems to us that this finding of a means of further enhancing the amount of liver fat should make easier the problem of investigating the source of this fat, for if any serious proportion of the liver fat in these livers comes from the fat depots, the quantity present is so great as to make its disappearance from the depots easily demonstrable. The difficulty hitherto has been that the amount of fat appearing in the liver has been quantitatively too small to make investigation of this problem in regard to the depots possible and to this problem we are now giving attention.

At the present stage it would be unwise to attempt to interpret the mechanism of the cystine action until further work has been carried out, for so far as we are aware, the only recorded finding of a similar nature was that of Curtis and Newburgh [1927], who recorded a single instance of fatty infiltration and severe necrosis in a rat receiving a diet containing 15 % caseinogen with 4 % cystine. In this connection it may be well to state that, although we have not yet carried out experiments on the action of choline in preventing the cystine effect, we have observed that, if the protein content of the diet is increased to 30 % with the fat remaining constant at 40 %, fat infiltration does not occur, which shows that an increased caseinogen intake can prevent not only the fat infiltration due to the high fat diet but also the superimposed effect of cystine.

We may add that one experiment has been carried out with each of the amino-acids lysine, glutamic acid, aspartic acid, serine, glycine and phenyl-alanine. Whilst deductions cannot be made with certainty from single experiments in this type of work, it may be mentioned that no effect either in increasing or decreasing the amount of liver fat has been as yet observed. These studies are being extended to the other amino-acids and related compounds.

SUMMARY.

1. Groups of rats have been fed on a diet containing caseinogen 5, fat 40, glucose 44, marmite 5 and salts 5, with and without the addition of cystine, such that each rat ingested from 3.25 to 80 mg. of cystine daily over periods of 14 to 21 days.
2. If all the results be pooled and expressed on the basis of the 100 g. rat, the average amount of fat in the livers of 45 control animals was 1.077 g., whereas that of the cystine-fed animals was 2.02 g., the amount of liver fat being doubled by the addition of cystine. This increase is caused by some 50 % increase in the percentage of fat in the liver, together with a 25 % increase in the liver weight.
3. Some abnormally high values were obtained in animals which received cystine, the highest being an animal in which the liver constituted 11.88 % of the body weight and contained 48.96 % of its fresh weight as fat, which corresponds to the presence in the liver of a 100 g. rat of 5.817 g. of fat.

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XLV. THE OXIDATION OF LECITHIN AND OTHER FATTY SUBSTANCES IN THE PRESENCE OF GLUTATHIONE.

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THE theory has frequently been advanced that the liver is intimately connected with oxidative processes involving the metabolism of fat in the body. The well-known desaturation theory, according to which a desaturation of fatty acids occurs in the liver giving a more oxidisable form of fat which is then passed on to the tissues, has been linked particularly with the work of Leathes and Meyer-Wedell [1909] and of Hartley [1909]. The validity of the desaturation theory and of the findings on which it rests have been questioned by Aylward *et al.* [1935] and by Turner [1930].

The possible relation of lecithin to fat metabolism has been made the subject of numerous investigations particularly by Bloor [1916], Artom [1931] and Sinclair [1932]. Jost [1931] showed that in perfusion experiments phosphatide was taken up by the liver, with the formation of ketone and an increase in the oxygen uptake of the organ, and concluded that the breakdown of fatty acids occurs through the intermediary of phosphatide.

In addition to its high lecithin content, the liver is also known to contain a large amount of the sulphydryl compound glutathione, which is thought to play an active part in the oxidation mechanisms in animal tissues, and which also has been found to be capable of bringing about the oxidation of fat [Hopkins, 1921; 1925]. A similar increase in the oxygen uptake of fats with other sulphydryl compounds was observed by Meyerhof [1923], whilst the effect of amino-acids was studied by Franke [1932] and by Page and Bülow [1935]. The latter workers extended previous investigations on the catalytic action of metallic salts and observed a marked increase in the rate of oxidation, particularly of kephalin, in the presence of iron.

The present investigation was suggested by a consideration of the fact that the liver is known to contain a readily oxidisable form of fat in the shape of lecithin and in addition a substance (glutathione) having oxidation-reduction capacities which had previously [Hopkins, 1921; 1925] been shown capable of bringing about the oxidation of fat. The experiments were devised with the purpose of determining whether the combination of fatty acid radicals in the lecithin molecule would render them more susceptible to oxidation than would be the case if they were exposed to the glutathione system either in the free state or as neutral fat.

EXPERIMENTAL.

The glutathione used in these experiments was prepared in the reduced form by the procedure of Pirie [1930]; a specimen in the oxidised condition was obtained by mild treatment with hydrogen peroxide by Schöberl's method [1931]. The phosphatides were freshly prepared according to the procedures used in a previous investigation [King, 1934]. Several specimens of purified fatty acids and glycerides were furnished by Dr A. D. Barbour of the Ontario

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Research Foundation. Our thanks are due to him, and to Dr C. S. Hanes of the same institution for the loan of a Barcroft apparatus.

Anaerobic oxidations. The experiments constituted an attempt to discover if, under any circumstances, glutathione was capable of oxidising lipid by transferring hydrogen atoms from the lipid to a reducible dye. For this purpose Thunberg tubes were employed. Hopkins's experiments with methylene blue, glutathione and lipid were repeated and his negative results confirmed.

o-Cresolindophenol, which decolorised immediately with reduced glutathione, was used in a series of experiments with lipid and oxidised glutathione. Glutathione solutions (5 mg. per ml. of buffer) and emulsions of lipid (10 mg. per ml. of buffer) at p_H 8.4 were made up fresh each day. (Triethanolamine was used as an emulsifying agent.) A solution of 1 : 2500 *o*-cresolindophenol was found to be of a suitable strength and to keep well if preserved in the dark. The test solutions for a typical experiment were set up as follows:

1 ml. emulsion (10 mg. fat) + 1 ml. buffer + 0.5 ml. *o*-cresolindophenol + 1 ml. glutathione solution (5 mg. G.S.S.G.¹).

The emulsion consisted in (1) of lecithin, in (2) of fatty substance other than lecithin and was replaced by water in a control experiment.

Lecithin induced the most rapid colour change. After lecithin came lysolecithin, kephalin and hydrolecithin followed by the fatty acids in the order of their iodine numbers. The triglycerides, even those with a comparatively high iodine number, decolorised the dye practically no faster than did glutathione alone (Table 1).

Table 1. *Decoloration of o-cresolindophenol by fatty substances with glutathione.*

Compound	Iodine number	Time for total decoloration min.
I. Liver lecithin	77	110
Egg lecithin	48	120
Kephalin	84	130
II. Lysolecithin	0	136
Hydrolecithin	0	188
Synthetic lecithin	0	264
III. Linolic acid	139	190
Oleic acid	81	256
IV. Linseed oil	120	216
Olive oil	81	256
V. Palmitic acid	0	280-320
Stearic acid	0	280-320
Tripalmitin	0	280-320
Distearin	0	280-320
Triolein	58	280-320
G.S.S.G.	—	280-320

Aerobic oxidations. The influence of glutathione upon the aerobic oxidation of lipoids was investigated by means of the Barcroft apparatus. Duplicate experiments were run as follows:

	(1)		(2)		(3)		(4)	
	Left cup	Right cup	Left cup	Right cup	Left cup	Right cup	Left cup	Right cup
2 ml. lecithin (20 mg.) in phthalate buffer	+	+	+	+				
2 ml. fatty acid (20 mg.) in phthalate buffer					+	+	+	+
1 ml. G.S.H. ² (8 mg.) in phthalate buffer		+		+				+
1 ml. phthalate buffer	+		+		+		+	

¹ G.S.S.G. = oxidised glutathione.

² G.S.H. = reduced glutathione.

The p_H in all experiments was 3.5¹ and the temperature 38°. Phthalate buffer was used throughout; a preliminary experiment with veronal buffer showed an inhibitory effect upon oxidation.

Several experiments were carried out in this way to test the comparative rates of oxygen uptake by lecithin, unsaturated fatty acids and a triglyceride. The results (Table II) indicated that oxygen was absorbed much faster by the

Table II. *Oxygen uptake of egg lecithin, fatty acids and triglyceride.*

	Iodine No.	O ₂ uptake (μl.)	
		220 min.	340 min.
20 mg. egg lecithin	65	340	440
20 mg. linolic acid	139	252	354
20 mg. oleic acid	80	48	62
20 mg. triolein	58	26	40

lecithin emulsions than by those of the fatty acids or of the neutral fat (triglyceride). The inference would seem justifiable that fat in the form of lecithin is more readily oxidisable under the action of glutathione than are other forms of fat.

A promising means of establishing whether or not the chemical combination of a fatty acid in the lecithin molecule leads to a more ready susceptibility to oxidation in the glutathione system was to compare the actual fatty acids, obtained from lecithin on hydrolysis, with the parent lecithin. Barium hydroxide hydrolyses were carried out on several different lecithins and the products of hydrolysis isolated according to the procedure of Channon and Chibnall [1927]. The results of a typical set of experiments are given in Fig. 1. The oxygen uptake of lecithin is roughly about four times that which would be expected from its iodine number in relation to the iodine numbers of its constituent fatty acids and their oxygen uptake.

Experiments upon brain lecithin and brain kephalin were also performed and the results are given in Fig. 2. In spite of the fact that the brain lecithin had a slightly lower iodine number than kephalin, its oxygen uptake was almost twice as great. As in the case of egg lecithin, the hydrolysis products of brain lecithin were found to have a much lower oxygen uptake than that of the parent lecithin in spite of their higher iodine number.

Lysolecithin and hydrolecithin displayed a very slow rate of oxygen uptake. When mixed with lysolecithin, unsaturated fatty acids absorbed oxygen somewhat more slowly than when exposed alone. This was probably due to the fact that there was less actual fatty acid present in the mixture, a proportion of the 20 mg. (the quantity of fatty substance invariably used) being practically non-oxygen-absorbing. The slow rate of aerobic oxidation of lysolecithin is in contrast to the fairly rapid rate of decolorisation of *o*-cresolindophenol in the anaerobic oxidations (Table I).

As was to be expected, no carbon dioxide was given off during the oxidations of the fatty substances.

It is possible to calculate the degree to which the fatty substances used in these experiments were oxidised, using as a criterion their iodine numbers on the basis of each atom of iodine being the equivalent of half an atom of oxygen.

¹ Hopkins emphasised the importance of the hydrogen ion concentration of his systems, pointing out that tissue oxidation took place most rapidly at p_H 7.4 and lipid oxidation at about p_H 3.5.

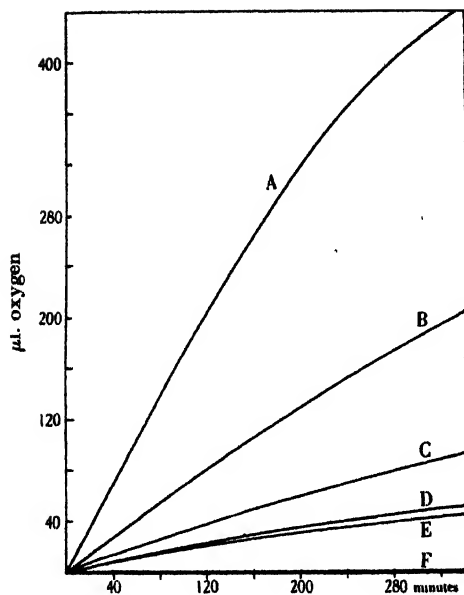


Fig. 1.

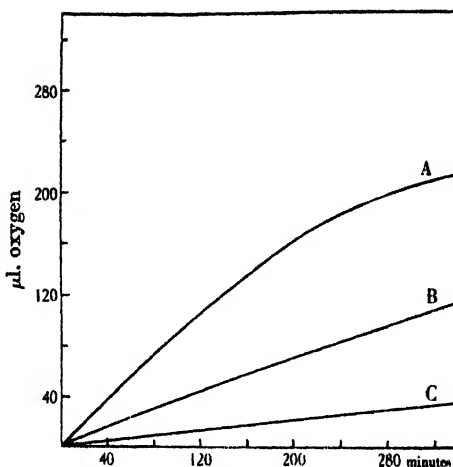


Fig. 2.

Fig. 1. Oxygen uptake of egg lecithin and its hydrolysis products: *A*, egg lecithin (Iodine No. 65); *B*, unsaturated fatty acids from hydrolysis (Iodine No. 101); *C*, Purified mixed fatty acids from hydrolysis (Iodine No. 85); *D*, constituents of lecithin mixed in molecular proportions; *E*, crude fatty acids from hydrolysis (Iodine No. 78); *F*, saturated fatty acids from hydrolysis.

Fig. 2. Oxygen uptake of brain lecithin, its hydrolysis products and brain kephalin. *A*, brain lecithin (Iodine No. 51); *B*, brain kephalin (Iodine No. 54); *C*, mixed fatty acids from brain lecithin (Iodine No. 65).

Table III. *Oxygen uptake of fatty substances with glutathione in relation to their iodine numbers.*

Compound	Iodine No.	Oxygen uptake in 340 min. μ l.	% of total theoretical oxygen uptake*
Egg lecithin	65.5	440	76.2
Crude fatty acids from hydrolysis of egg lecithin	78	52	7.6
Purified fatty acids from hydrolysis of egg lecithin	85.4	92	12.2
Unsaturated fatty acids from hydrolysis of egg lecithin	101	200	22.5
Constituents of egg lecithin mixed in molecular proportions	—	54	12.2
Brain lecithin	51	216	48.0
Purified fatty acids from hydrolysis of brain lecithin	65	36	6.3
Brain kephalin	54	112	23.5
Linolic acid	139	354	28.9
Triolein	58	40	7.8
Oleic acid	93.4	62	7.5

* Calculation performed as follows: e.g. linolic acid, iodine number 139. 100 mg. linolic acid \equiv 139 mg. iodine \equiv 8.76 mg. oxygen \equiv 6.12 ml. Actual oxygen uptake of 100 mg. linolic acid = $\frac{0.354}{20} \times 100 = 1.77$ ml.; % of theoretical oxygen uptake = $\frac{1.77 \times 100}{6.12} = 28.9$.

Table III shows that lecithin absorbed a very considerable fraction of its theoretical oxygen equivalent; contrasted with this are the much smaller figures for the unsaturated acids and the other forms of fat.

DISCUSSION.

Careful microscopic examination of the emulsions used in these experiments revealed the fact that at p_{H} 3.5, those of lecithin and free fatty acids contained particles of approximately the same size. (It is also of interest to note that Hopkins [1925] stated that the size of particles had no influence on the rate of oxygen uptake in the Barcroft apparatus.) It would therefore appear that the extra oxygen uptake of lecithin over that of its constituent fatty acids is a chemical phenomenon and that the configuration of the lecithin molecule renders it peculiarly susceptible to oxidation in the glutathione system. The mere presence of phosphate and choline in an emulsion of fatty acid induces no further oxygen uptake than would be expected from the oxidation of fatty acid alone. That the presence of phosphate in the lecithin molecule is not solely responsible for its ease of oxidation, but that choline also plays a part, may be inferred from the fact that lecithin (which contains choline) is more rapidly oxidisable than kephalin, which contains phosphorus but has amino-ethyl alcohol instead of choline for its base.

Page and Bülow [1935] found kephalin to be more rapidly oxidised than lecithin when iron or copper was present as catalyst (whereas in the present experiments with glutathione the reverse was the case). Tests for iron in the mixtures used revealed the presence of about 6 γ Fe [Page and Bülow used 0.3 mg.], most of it being in the glutathione. The addition of small amounts of extra iron was without effect. Varying amounts of cyanide up to 1 mg. failed to alter the oxygen uptake with any of the lipid materials tried, although larger amounts (5–20 mg.) caused a graded inhibition with almost complete suppression of the oxygen consumption when more than 20 mg. of cyanide were present. 1 mg. of cyanide is more than sufficient to suppress any activity which might be due to the 6 γ of iron. It is therefore improbable that iron acting catalytically affected the rate of oxygen uptake or that it was concerned in the glutathione catalysis of the aerobic oxidation of the fatty substances. Meldrum and Dixon [1930] detected no difference between "impure" and crystalline glutathione in the oxygen uptake induced with linolic acid, although the crystalline substance required the presence of iron and an "activator" to bring about the oxidation of protein.

In aerobic fat oxidation by glutathione the sulphydryl-disulphide relation is apparently not involved [Allot, 1923; Meldrum and Dixon, 1930]. The 8 mg. of reduced glutathione used in the experiments should require 146 μ l. of oxygen for conversion into the disulphide, but in blank experiments no evidence of this transformation could be obtained, and in the fat oxidations the glutathione was always present in the reduced form at the termination of the experiment. Substitution of oxidised for reduced glutathione failed to elicit an oxygen uptake in excess of that in the controls. In all experiments with lecithin and in many of those with unsaturated fatty acids the oxygen absorbed was greatly in excess of the oxygen equivalent of the glutathione present, which would appear to have acted in a catalytic fashion to influence the rate of oxygen uptake.

SUMMARY.

1. A comparison of the rates of oxidation in the presence of glutathione, of lecithin, kephalin, neutral fats and fatty acids, including those obtained from lecithin on hydrolysis, has been made.

2. Under anaerobic conditions at a slightly alkaline reaction, lecithin appears to be much more rapidly oxidised by means of oxidised glutathione than is neutral fat or fatty acid.

3. At an acid reaction the oxygen uptakes of lecithin and reduced glutathione are greater than that of the neutral fat or fatty acids tested and also greater than that of the fatty acids obtained from lecithin on hydrolysis.

4. The oxygen uptake of lecithin is about four times that which would be expected from a consideration of its iodine number in relation to the iodine number of its constituent fatty acids and their oxygen uptakes.

5. Calculated on the basis of iodine numbers the oxygen uptake of lecithin is a much higher percentage of the theoretical than is the case with unsaturated fatty acids.

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XLVI. COMPARATIVE ACTIVITIES OF COMPOUNDS OF THE ANDROSTERONE-TESTOSTERONE SERIES.

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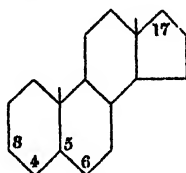
I. Introduction.

THE first isolation of a crystalline substance with male hormone properties was carried out by Butenandt [1931], who obtained from male urine a small amount of the substance to which he subsequently gave the name androsterone. Later, he was able to prepare larger quantities and to suggest that the substance was a fully hydrogenated *cyclopentenophenanthrene* derivative with a hydroxyl group in position 3 and a keto-group in position 17 [Butenandt, 1932; 1933]. Ruzicka *et al.* [1934] were able to confirm this hypothesis by the chemical preparation of androsterone from *epicholesterol*, thus determining the configurations of carbon atoms 3 and 5.

In the meantime evidence had been accumulating that the male hormone activity of male urine differed quantitatively from that of testicular extracts, notably in its smaller activity per capon unit on the seminal vesicles of castrated rats (see Dingemans *et al.* [1935] for refs.), and it was soon evident that androsterone was not the chief active principle of testicular extracts [David and Freud, 1935; Callow and Deanesly, 1935, 1]. The search for other male hormone substances therefore continued. Butenandt and Dannenbaum [1934] had previously isolated from male urine an unsaturated substance closely related to androsterone and having about one-third of its activity, *transdehydroandrosterone*. Ruzicka was led to believe that the testicular hormone itself, by analogy with progesterone, might be an unsaturated compound related to androsterone [Ruzicka and Wettstein, 1935, 1]; having produced *transdehydroandrosterone* ($\Delta^5,6$ -*trans*-3-hydroxy-17-keto-androstene) by degradation of cholesterol, he set about the systematic production of other compounds of the series. While these developments were taking place, David *et al.* [1935] had obtained from testis a crystalline substance having high activity on both capons and rats, and apparently of sterol type, to which they gave the name testosterone. On the basis of his biological tests of the substances prepared by Ruzicka, Tschopp [1935, 1] was able to suggest that the testosterone of Laqueur and his co-workers might be the androstenedione already prepared by Ruzicka, or possibly the corresponding 17-hydroxy-3-keto-compound. David [1935] then showed that oxidation of testosterone yielded the unsaturated diketo-compound and it seemed certain that testosterone must be Δ^4 -17-hydroxy-3-keto-androstene. The next step in this remarkable story was the report by Butenandt and Hanisch [1935, 1, 2] of the artificial production of this compound from dehydroandrosterone and its identification with Laqueur's testosterone. Simultaneously, Wettstein [1935], and soon afterwards Ruzicka and Wettstein [1935, 2], published details of the completion of their projected synthesis of Δ^4 -17-hydroxy-3-keto-androstene.

Allowing for transposition of the hydroxyl and ketone groups at positions 3 and 17, for *cis* and *trans* configurations of the 3-hydroxyl groups, for the diketone and the dihydroxy-compounds, but not for differences in configuration of the 17-hydroxyl, there are twelve possible derivatives in the series, six from androstane and six from androstene. Of these twelve, ten have so far been produced artificially from cholesterol. (See Table I.) The remaining two involve the

Table I.			
Androstane derivatives (saturated)		Androstene derivatives (unsaturated: Δ^4 or $\Delta^{5,6}$)	
	3- <i>cis</i>	3- <i>trans</i>	
3-hydroxy-17-keto-	Androsterone	<i>trans</i> Androsterone	Dehydroandrosterone (not known)
3:17-dihydroxy-	Androstanediol	<i>trans</i> Androstanediol	Androstenediol (not known)
17-hydroxy-3-keto-		3-Keto-androstanol	Testosterone
3:17-diketo-		Androstanedione	Androstenedione



Androstane

technical difficulty of securing a *cis*-3-hydroxyl with a 5 : 6 double linking. Of the ten now available, androsterone, *trans*androsterone, *trans*dehydroandrosterone, testosterone and androstenedione have been referred to above. The others include *cis*androstanediol [Ruzicka, Goldberg and Meyer, 1935, 2; Butenandt and Tscherning, 1935], *trans*androstanediol [Ruzicka, Goldberg and Rosenberg, 1935], androstanedione [Butenandt and Tscherning, 1934, 2; Ruzicka, Goldberg and Meyer, 1935, 1], and *trans*androstenediol [Ruzicka and Wettstein, 1935, 1]. Further, Ruzicka and his co-workers have made various esters of the compounds [see Ruzicka, Wettstein and Kägi [1935] in addition to papers cited above], and also derivatives methylated or ethylated at position 17, including methyltestosterone.

The biological properties of androsterone were briefly examined by Butenandt and his co-workers after their initial isolation of the substance (see also Tscherning [1933]), and have since been investigated in detail by Ruzicka, Goldberg *et al.* [1934], Butenandt and Tscherning [1934, 1], Korenchevsky [1935], David and Freud [1935], Callow and Deanesly [1935, 1], Callow and Parkes [1935], Greenwood *et al.* [1935], Tschopp [1935, 2] and Korenchevsky and Dennison [1935, 1]. Androstanediol has been investigated by Tscherning [1934], and by Butenandt and Tscherning [1935], Ruzicka, Goldberg and Meyer [1935, 2], David and Freud [1935], Callow and Deanesly [1935, 2] and Korenchevsky and Dennison [1935, 2].

The biological activities of other compounds of the series, including testosterone, have been examined by Ruzicka and his co-workers in the papers referred to above, by Tschopp [1936] in great detail and to a less extent by Butenandt, in the papers referred to above, and by Parkes [1935]. Reference to their findings is made in the appropriate place below.

The work described below was undertaken as a study of the influence on biological activity of slight changes in molecular structure, for which the

androsterone-testosterone series of male hormone compounds offers scope unrivalled in endocrinology. At the same time it was hoped to find out to what extent compounds known at present, especially testosterone, exhibit the biological properties attributable to the testicular hormone or hormones.

II. *Material and technique.*

The comparative biological activities of the following compounds have been tested:

Androsterone
Androstanediol
*trans*Dehydroandrosterone
*trans*Androstenediol
Testosterone
Androstenedione
Methylandrostanediol
Methyl-*trans*androstenediol
Methyl-3-keto-androstanol
Methyltestosterone
Testosterone benzoate

These compounds were all prepared by Prof. L. Ruzicka and Messrs Ciba.

All the substances were dissolved in arachis oil for injection. Their solubility varied greatly. Androsterone and *trans*dehydroandrosterone are readily soluble at 10 mg./ml.; heating is required to make the solution, but the material does not separate at room temperature. Androstanediol, *trans*androstenediol and methyl-*trans*androstenediol do not stay in solution at room temperature even at 2.5 mg./ml., and heating immediately before injection is necessary if concentrations of more than about 1.5 mg./ml. are being used. Methylandrostanediol is much more soluble. The other compounds remain in solution at the highest concentrations we have made, 5 mg./ml.

Capon tests. The work on the capon comb was carried out as described by Callow and Parkes [1935] and the results were obtained by reference to the standard curve given by Greenwood *et al.* [1935]. Each series of tests was carried out on groups of capons of the same hatch and caponised together. In each experiment a control group received a total dose of 1 mg. androsterone per bird and the activity of the simultaneously tested substance was calculated in comparison with the result on the control group. A total of 1 mg. androsterone gives an average increase in comb size (L+H) of between 6 and 10 mm., depending on the sensitivity of the birds. The doses of the other compounds were adjusted to give a response of similar magnitude before a final comparison was made. In this way the effect of variation in response and of any difference in the slope of the dose/response curves for the various compounds (such as is found in rats) was minimised.

Rat tests. The rats were injected subcutaneously. We have evidence that the volume of oil injected influences the response appreciably and, with one or two exceptions, the volume of oil solution injected daily was standardised at 0.2 ml. The technique of assay on rats was exactly as described by Callow and Deanesly [1935, 1], *i.e.* immature males were castrated at about 40–50 g. body weight and used not less than a month later, the total dose of hormone being given over 10 days. The organs were weighed from 70 % alcohol after fixation in Bouin's fluid—a technique employed by us for many years (see *e.g.* Brambell and Parkes [1929]). There is no doubt that the use of rats immediately after castration, before glandular atrophy has set in, greatly increases the apparent response to

treatment, and this fact may explain some of the discrepancies in the results of various workers. Neither the weight of rat nor the time (above 1 month) after castration at injection was strictly standardised, but we have failed to obtain any evidence that within reasonable limits these factors have any regular influence on the result. Groups of 5 rats were used for each test. Many of the tests were duplicated after an interval, giving a total of 10 rats on each dose.

III. Activity on the capon comb.

Table II gives the results obtained with the capon comb test. In addition to preliminary trials, two tests were made with each compound, except *trans*-androsterone (for which the single result is copied from Callow and Deanesly [1935, 1]) and *trans*androstenediol, on which a third test was made. Apart from

Table II. Activity on the capon comb.

Compound	Amount (γ) required to \equiv 100 γ androsterone		International unit as tested on capons (approx.) γ	International units per mg. (approx.)
	1st test	2nd test		
A. Androsterone	100	100	100	10
B. <i>trans</i> Androsterone	700	—	700	1.5
C. Androstanediol	33	30	33	30
D. Methylandrostanediol	27	22	25	40
E. Methyl-3-keto-androstanol	24	23	24	40
F. <i>trans</i> Dehydroandrosterone	310	295	300	3
G. Androstenedione	110	97	100	10
H. <i>trans</i> Androstenediol	175	250, 280	235	4
J. Methyl <i>trans</i> androstenediol	160	150	155	7
K. Testosterone	16	18	17	60
L. Methyltestosterone	70	90	80	12

*trans*androstenediol, there is no serious discrepancy in the duplicate tests. Several of the results, however, are significantly different from those obtained by Tschopp [1936]. This difference may be due to the fact that his comparisons were made at a level of response given by 70 γ androsterone daily, whereas ours were made at a level given by 200 γ daily.

The following observations may be made on the data given in Table II.

(a) The only compounds less active than androsterone are those having a *trans*-3-hydroxyl group. Such a group is therefore specially unfavourable to activity on capons. Oxidation to a 3-keto-group (*trans*dehydroandrosterone to androstenedione) or conversion into a *cis*-3-hydroxyl (*trans*androsterone to androsterone) increases the activity considerably.

(b) The fact that methylandrostanediol and methyl-3-keto-androstanol are of similar activity suggests that the oxidation of the *cis*-3-hydroxyl group to a 3-keto-group has little effect on activity, a conclusion in keeping with Tschopp's data for androsterone and androstanediol. It may be supposed therefore that the so far unknown *cis*dehydroandrosterone and *cis*androstenediol will have activities on capons similar to those of androstenedione and testosterone respectively.

(c) Reduction of the 17-keto-group to a hydroxyl group increases the activity, slightly (*trans*dehydroandrosterone to *trans*androstenediol) or greatly (androsterone to androstanediol, and androstenedione to testosterone).

(d) The effect of unsaturation on the activity on capons is inconstant. Comparison of *trans*androsterone with *trans*dehydroandrosterone suggests an increase of activity by unsaturation. Tschopp's corresponding data support this, but his figures for androstanediol and androstenedione and for *trans*androstanediol

and *trans*androstenediol do not imply any effect of unsaturation on activity on capons.

(e) The introduction of a 17-methyl group slightly increases activity on the capon comb, except for the anomaly of methyltestosterone which is much less active than testosterone.

Activity of testosterone benzoate. Androsterone benzoate has a delayed and prolonged action on the capon comb [Callow and Deanesly, 1935, 1; Callow, 1936], but the ultimate amount of growth produced may be even greater than that caused by a similar amount of free hormone. Testosterone benzoate however was found to have no appreciable activity. A total dose of 1 mg. to each of 5 capons gave no detectable comb growth within a month.

IV. Activity on the prostate and seminal vesicles of the castrated rat.

Nature of dose/response curves. The results of the rat experiments are summarised in Tables III and IV and shown graphically in Figs. 1-4. In these

Table III. Activity of non-methylated compounds on the castrated rat.

Compound	Total dose (mg.)	No. of rats	Average weight of prostate (mg.)	Average weight of seminal vesicles (mg.)
A. Androsterone	3.5	10	37	8
	6.0	11	54	12
	10.0	10	92	22
	15.0	10	135	23
	20.0	10	141	32
C. Androstenediol	1.5	5	54	16
	2.5	10	73	30
	5.0	10	96	34
	10.0	10	168	79
F. <i>trans</i> Dehydroandrosterone	10.0	5	19	6
	20.0	5	24	9
G. Androstenedione	2.5	5	58	22
	5.0	10	78	33
	10.0	10	150	58
H. <i>trans</i> Androstenediol	5.0	10	59	28
	10.0	10	104	82
K. Testosterone	0.6	5	33	10
	2.0	5	61	27
	4.0	5	96	47
	6.0	5	136	84
	8.0	5	156	99

Table IV. Activity of methylated compounds on the castrated rat.

Compound	Total dose (mg.)	No. of rats	Average weight of prostate (mg.)	Average weight of seminal vesicles (mg.)
D. Methylandrostanediol	2.5	5	40	27
	5.0	5	151	59
	7.5	5	201	78
E. Methyl-3-keto-androstanol	2.5	5	83	23
	7.5	5	230	136
J. Methyl <i>trans</i> androstenediol	2.5	5	30	9
	5.0	5	105	50
	7.5	5	116	65
L. Methyltestosterone	2.0	5	75	32

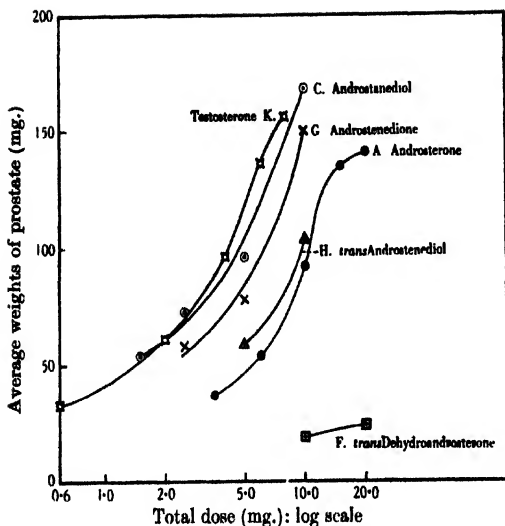


Fig. 1.

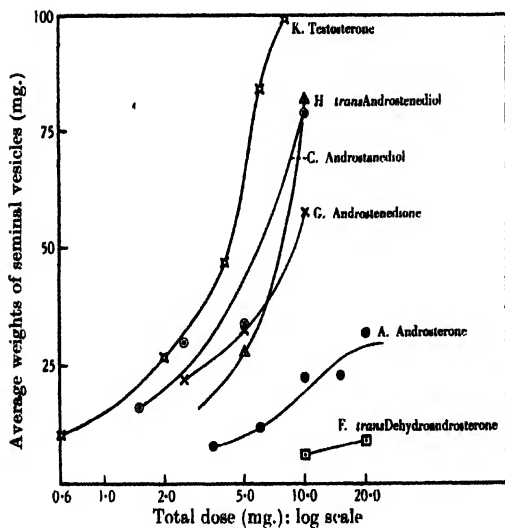


Fig. 2.

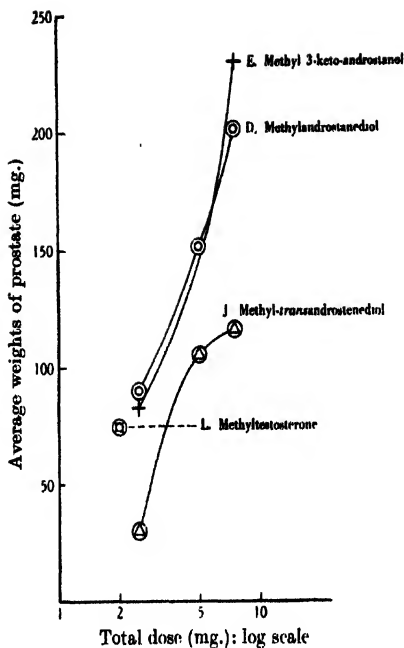


Fig. 3.

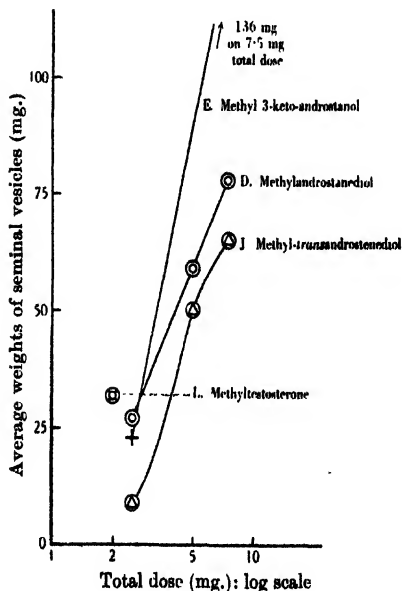


Fig. 4.

Fig. 1. Response of the prostate of the castrated rat to male hormone compounds (non-methylated).

Fig. 2. Response of the seminal vesicles of the castrated rat to male hormone compounds (non-methylated).

Fig. 3. Response of the prostate of the castrated rat to male hormone compounds (methylated).

Fig. 4. Response of the seminal vesicles of the castrated rat to male hormone compounds (methylated).

graphs the ordinates are logarithmic, and in 3 of the 4 experiments for which there are most data (androsterone and testosterone, both prostate and seminal vesicles) the results suggest strongly that the response bears not a linear but a sigmoid relationship to the logarithm of the dose. The curves given by androstanediol and androstenedione can be considered as the middle parts of sigmoid curves, and the same applies to methylandrostanediol and methyl*trans*-androstanediol. For the remaining four compounds the data are inadequate to show the nature of the dose/response curve. Further analysis of these curves and of the variability of the response will be made elsewhere.

Comparative activities of compounds. Testosterone is the most active of any of the non-methylated compounds. From our results, this superiority is well marked on the seminal vesicles, but insignificant on the prostate. On both it is much less than shown by Tschopp's results. Further, we find that methyl-3-keto-androstanol is more active than testosterone on both prostate and seminal vesicles, and methylandrostanediol is more active on the prostate, though both were found by Tschopp to be less active. On both the prostate and seminal vesicles the non-methylated compounds have the following order of descending activity, androstanediol, androstenedione, *trans*androstanediol, androsterone, *trans*dehydroandrosterone, except that the curve for the response of the seminal vesicles to *trans*androstanediol intersects that for the response to androstenedione. Relative to the other compounds however androsterone is much less active on the seminal vesicles than on the prostate, whilst *trans*androstanediol is more active on the seminal vesicles than on the prostate. This point will be dealt with more fully below, but meanwhile it may be noted:

(a) That taking androsterone as the standard substance, comparisons of activity on the seminal vesicles will give very different results from those obtained by comparisons on the prostate. Thus (Figs. 1 and 2) testosterone is about 10 times more active than androsterone when tested on the seminal vesicles, but only 2-5 times when tested on the prostate. *trans*Androstenediol is an extreme case: it is only slightly more active on the prostate than androsterone, but it is 4-5 times more active on the seminal vesicles.

(b) Differences in the slope of the dose/response curves for the different substances make it, in some cases, almost impossible to give a definite ratio for their relative activities. This applies whether the relative amounts of two substances required to produce the same degree of response are compared or the relative responses produced by the same amount of two substances. Thus on the prostate, 1 mg. of testosterone \equiv 4 mg. of androsterone, but 5 mg. \equiv 12 mg. and 6 mg. \equiv 20 mg.

Similarly, from the above results, the relative activities of androstanediol, *trans*androstanediol and androstenedione depend very much on the points at which the comparison is made. In these circumstances it is difficult to see how one substance can be equated with another and in particular to see how androsterone can serve in a rat test as a standard for substances other than androsterone or androsterone-containing extracts. For these reasons we refrain from trying to construct any table of relative activities of the various compounds on rats.

This difference in slope of the dose/response curves for different substances does not seem hitherto to have been specifically noted, probably because such an extensive group of hormones as the androsterone-testosterone series possessing similar biological activities has not hitherto been available. A comparative investigation of oestrone, oestradiol and oestriol from the point of view of slope of dose/response curves seems to be indicated. Hill *et al.* [1934]

observed a difference in slope of the dose/response curves for the ovulation-producing activity of hypophyseal and urine extracts, but such extracts are so complex as to be scarcely comparable with crystalline substances.

The following tentative conclusions may be drawn about the effect of molecular configuration on male hormone activity in rats.

(a) The *trans*-configuration of the 3-hydroxyl group is obviously unfavourable to activity in *trans*androsterone, but is not incompatible with good activity in *trans*androstenediol, especially on the seminal vesicles. The *cis*-3-hydroxyl and the 3-keto-group confer about equal activities on the compound (methyl-androstanediol and methyl-3-keto-androstanol). Tschopp's data on androsterone and androstanedione suggest a similar conclusion.

(b) Reduction of the 17-keto-group to hydroxyl greatly enhances activity, as shown by three pairs of compounds, androsterone and androstanediol, *trans*-dehydroandrosterone and *trans*androstenediol, and androstenedione and testosterone.

(c) The effect of unsaturation is rather uncertain. The data are inadequate for a comparison of *trans*androsterone with *trans*dehydroandrosterone. According to Tschopp, *trans*androstenediol is more active than *trans*androstanediol and androstenedione is more active than androstanedione. On the other hand, in our experience, methyl-3-keto-androstanol is of the same order of activity as methyltestosterone.

(d) In the three pairs of compounds available for comparison, the introduction of a methyl group increases the activity appreciably.

Ratio of prostate/seminal vesicle growth. Callow and Deanesly [1935, 1] showed that androsterone caused an abnormal ratio of growth between prostate and seminal vesicles, the latter being small for the size of prostate. They also showed [1935, 2] that androstanediol produced a normal growth relation between the two organs. These results have been confirmed by Korenchevsky and Dennison [1935, 1, 2]. Of the compounds dealt with above, the data for *trans*dehydroandrosterone are inadequate, and with methyl-3-keto-androstanol and androstenedione the ratio is fairly normal. From Fig. 5 it will be seen that testosterone given for 10 days seems to produce larger seminal vesicles in relation to the size of prostate than are found in the normal rat, whilst with *trans*androstenediol the abnormality of the ratio is very marked. This may be evidence that testosterone is not the only male hormone produced by the testis. It should be noted that these new data about the prostate/seminal vesicle ratio in experimental rats relate only to glands in the early stages of growth. Methylation, judging from the results on methylandrostanediol and methyl*trans*androstenediol (Fig. 6) seems slightly to decrease activity on seminal vesicles relative to activity on the prostate.

With the available information few conclusions can be reached about the effect of molecular configuration on the prostate/seminal vesicle ratio. In two pairs of compounds, reduction of the 17-keto-group to hydroxyl increases activity on the seminal vesicles relative to that on the prostate (androsterone-androstanediol, androstenedione-testosterone), but oxidation of the 3-hydroxyl to a keto-group does not affect the ratio.

Oestrone given over short periods is known to have a stimulating action on the fibro-muscular tissue of the seminal vesicles, with little effect on the prostate, so that it might have been anticipated that the capacity of a male hormone compound to produce abnormally large seminal vesicles relative to the prostate would be correlated with the degree of oestrogenic activity of the compound (see Deanesly and Parkes [1936]). It is impossible however to detect any obvious

correlation between these two properties; testosterone, for instance, is only very weakly oestrogenic.

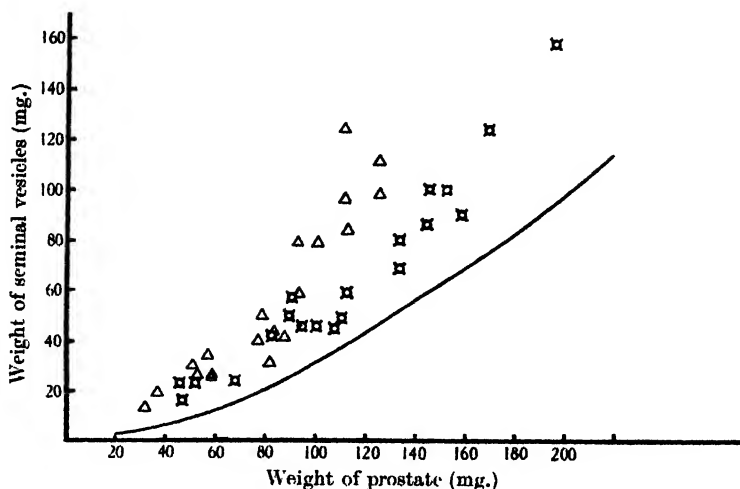


Fig. 5. Relation between growth of the prostate and of the seminal vesicles.

— Normal growth of seminal vesicles in relation to the prostate.
 Δ *H. transAndrostenediol*. \square *K. Testosterone*.

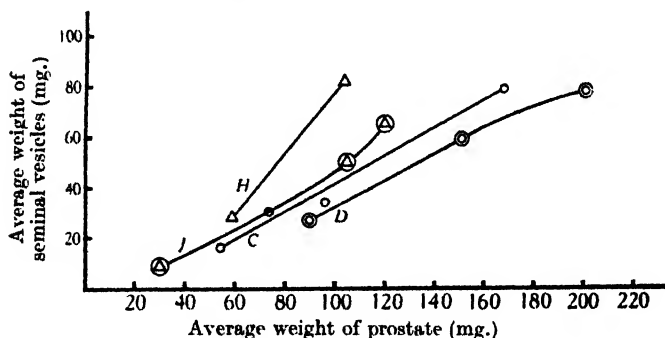


Fig. 6. Effect of methylation of a compound on relation between growth of prostate and of seminal vesicles.

\circ *C. Androstenediol*. \odot *D. Methylandrostenediol*.
 Δ *H. transAndrostenediol*. \odot *J. Methyl-transandrostenediol*.

V. Activity on rats in relation to activity on capons.

It has been mentioned above that extracts of male urine were found to be much less active on rats, per capon unit, than testicular extracts, and that the same applied to androsterone. Callow and Deanesly [1935, 2] found that androstenediol also had the same low activity on rats per capon unit as androsterone and thus lacked at least one characteristic property of testis extracts [cf. Korenchevsky *et al.* 1935]. It was anticipated that testosterone would be similar in this respect to testicular extracts, but our evidence, so far, points to the contrary. In Figs. 7 and 8 are shown the dose/response curves, of prostate and seminal vesicles respectively, for the various compounds, the abscissae being logarithmic scales of capon units (see Table I). From Fig. 7 it

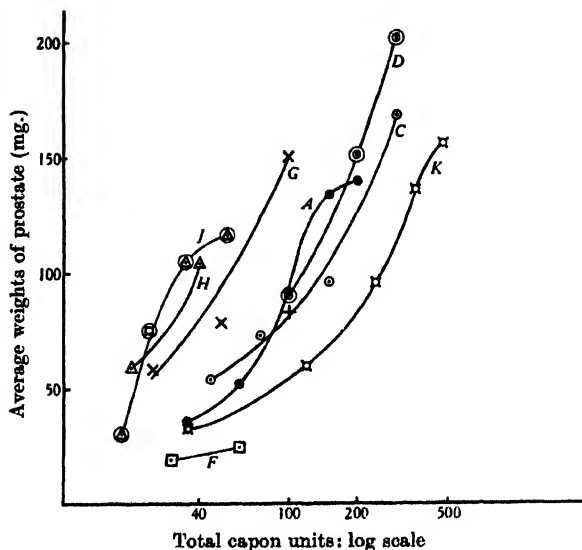


Fig. 7. Activities of various compounds on the prostate of the castrated rat in relation to their activities on the capon comb.

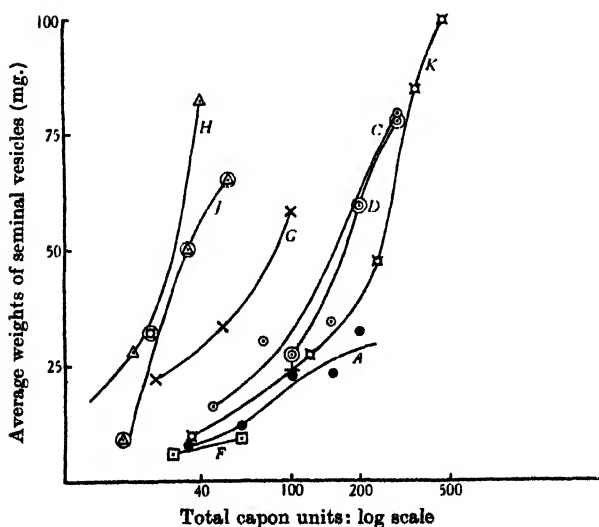


Fig. 8. Activities of various compounds on the seminal vesicles of the castrated rat in relation to their activities on the capon comb.

Explanation of symbols in Figs. 7 and 8:

- | | |
|----------------------------------|---|
| • A. Androsterone. | × G. Androstenedione. |
| ○ C. Androstanediol. | △ H. <i>trans</i> Androstenediol. |
| ⊙ D. Methylandrostanediol. | ⊕ J. Methyl- <i>trans</i> androstanediol. |
| + E. Methyl-3-keto-androstanol. | □ K. Testosterone. |
| □ F. Methyl-Dehydroandrosterone. | ⊕ L. Methyltestosterone. |

will be seen that, per capon unit, only four of the compounds have higher activities on the prostate than androsterone and that testosterone has appreciably less activity. Of the four more active ones, the two *trans*androstenediols occupy the position because of their low activities on capons (due to the *trans*-3-hydroxyl group), not because of marked activities on rats, and it is possible that the high activities per capon unit of these two compounds are not of much significance. Methyltestosterone is anomalous owing to the curious effect of methylation of testosterone on activity on capons.

All four of the compounds however are unsaturated and it is possible to infer that high activity per capon unit on the prostate is restricted to unsaturated compounds. Certainly Tschopp's figures for androstanedione and androstenedione, for which the capon activities are similar, provide a striking demonstration of the greater activity per capon unit of the unsaturated compound on rats. The four compounds whose activities per capon unit on the prostate are no greater than that of androsterone have no single feature in common and it is impossible at this stage to say what determines low activity per capon unit.

As regards the seminal vesicles, only *trans*dehydroandrosterone, of the compounds examined, had as low an activity per capon unit as androsterone. Testosterone, over the range at which comparison could be made, was very little better than androsterone, but androstanediol and its methylated derivative were slightly more active. As with the prostate however the highest activities per capon unit were shown by the four unsaturated compounds: androstenedione, *trans*androstenediol, methyl*trans*androstenediol, and methyltestosterone. The second of these appears to be ten times as active per capon unit as androsterone. The same general remarks on this varying activity per capon unit apply to the seminal vesicles as well as to the prostate.

SUMMARY.

1. The male hormone activities on capons and castrated rats of eleven compounds of the androsterone-testosterone series have been examined in relation to their molecular configurations and have been compared with the known activities of testis and urine extracts.

2. Testosterone, on both capons and rats, is far the most active of the three substances so far prepared from natural sources. It is also more active than any of the non-methylated "artificial" compounds. Differences in the slope of the dose/response curves on rats for the different substances make any definite figures for the relative activities of the various compounds unsatisfactory, but testosterone may be said to be about 6 times as active on capons, 2-5 times as active on the prostate and 10 times as active on the seminal vesicles of castrated rats as androsterone. Most of the other compounds show intermediate degrees of activity. The most active of all the compounds we have examined was methyl-3-keto-androstanol, i.e. methyl-dihydrotestosterone.

3. The following conclusions may be drawn with reference to the effect of molecular structure on male hormone activity:

(a) A *trans* configuration of the 3-hydroxyl group is especially unfavourable to activity on the capon comb (compare compounds A and B, Table II). It may be unfavourable to activity on the prostate and seminal vesicles of the castrated rat (as with *trans*androsterone), but is compatible with high activity (*trans*-androstenediol). Oxidation of the *cis*-3-hydroxyl group to a keto-group has little effect on activity, either on rats or capons (compare D and E, Tables II and IV, and Figs. 3-4).

(b) Reduction of a 17-keto-group to hydroxyl increases activities on both capons and rats (compare compounds A and C, F and H, and G and K, Tables II and III and Figs. 1 and 2), especially on the seminal vesicles of the latter.

(c) Unsaturation (Δ^4 or $\Delta^{5,6}$) may increase activity on capons (compare compounds B and F, Table II) but this does not apply to all compounds, for instance androstenedione and androstanedione [Tschopp, 1936]. The effect on activity on the rat is also inconstant.

(d) The introduction of a 17-methyl group increases activity on capons slightly in two compounds (compare C and D, H and J, Table II). In the case of testosterone it much decreases activity. Methylation enhances activity on rats, especially activity on the prostate, in all three pairs of compounds available for comparison.

4. *trans*-Androstenediol and to a less extent testosterone produce abnormally large seminal vesicles relative to the prostate (Fig. 5). The relative growths caused by compounds other than androsterone are fairly normal. Methylation somewhat decreases the growth of the seminal vesicles relative to that of the prostate (Fig. 6).

5. It is known that the rat activity of androsterone per capon unit is less than that of testicular extracts, so that it is curious that testosterone should have even less activity on the prostate per capon unit than androsterone (Fig. 7) and only slightly more on the seminal vesicles over the range where comparison is possible (Fig. 8). High activity per capon unit on rats is restricted to four other unsaturated compounds.

6. In view of the above conclusions, and also since testosterone is only very slightly oestrogenic [Deanesly and Parkes, 1936], it seems likely that this compound alone cannot account for the whole endocrine activity of the testis.

It is possible that an accessory substance as postulated by Laqueur and his co-workers [David *et al.*, 1935] is necessary to increase the activity of testosterone or that a further hormone is involved.

Our very best thanks are due to Prof. Ruzicka and Messrs Ciba for their most generous action in placing the various compounds at our disposal. We would also thank Dr K. Miescher and Dr E. Tschopp for allowing us to see unpublished manuscripts and for giving us much general information.

To Dr R. K. Callow we are deeply indebted for his constant advice and for much help in the preparation of this paper.

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XLVII. A STUDY OF THE CHEMICAL NATURE OF VITAMIN B₆ AND METHODS FOR ITS PREPARATION IN A CONCENTRATED STATE.

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THE term vitamin B₆ has been given by György [1934] to that part of the vitamin B₂ complex which is responsible for the cure of the specific dermatitis developed by young rats fed on a vitamin B-free diet supplemented with purified vitamin B₁ and lactoflavin. The object of this paper is to give an account of the study which has been made of this dietary factor.

PART I. EXTRACTION OF VITAMIN B₆.

The method of assaying vitamin B₆ was described by György [1935, 1]. It consists of finding the minimum daily dose of the test material needed to cure the rat of the specific "acrodynia-like" dermatitis. The amount necessary is defined as one unit. This method has been used in all our experiments and proved to be satisfactory. The biological tests are shown in Table I, the experimental details of the extraction of the vitamin being set out in the experimental section.

Previously György [1935, 1] had shown that fresh fish muscle was a good source of vitamin B₆. The results obtained with salmon, haddock and herrings are shown in Table I. As aqueous extracts of fish muscle contain relatively small amounts of solid material in comparison with aqueous extracts of yeast or liver (from which the water-soluble vitamins are often prepared), it was thought that fish would prove an excellent source for the preparation of concentrates. With this object in view both salmon and haddock muscle were extracted by boiling water; but with both materials less than 25% of the vitamin was found to be extracted (Table I, Exps. 2, 3 and 4).

Attempts were next made to extract the vitamin by means of alcohol, but again no more than 25% of the vitamin could be obtained in solution. In the first place, extraction with cold alcohol was tried. This proved unsuitable, and in a later experiment minced herring muscle was boiled under a reflux condenser with 50% alcohol containing 1% HCl. Unfortunately a similar result was obtained (Exps. 5 and 6).

As it appeared to be very difficult to get the vitamin into solution in anything approaching quantitative amounts by simple extraction, autolysis of the tissue was next tried. This method gave variable results. If only toluene were used as an antiseptic, 50-70% of the vitamin was obtained in solution, but the autolysis mixture developed a putrid smell, indicating that some bacterial decomposition had occurred. If chloroform in addition to toluene were used bacterial action was prevented, but only 25% of the activity was recovered in solution (Exps. 7, 8 and 9).

In order to ascertain if it were possible to obtain all of the vitamin in solution after the tissue had been completely broken down, herring muscle was hydrolysed by the action of papain. In this manner 75 % of the vitamin was recovered in solution (Exp. 10).

Table 1.

Exp.	Material tested	Amount fed	Healing of acrodynia	Weekly increase in g.	% of activity extracted
1	Fresh salmon	0.5 g.	Yes	8	
	Haddock	0.5 g.	Yes	3	
	Fresh herring	0.5 g.	Yes	6	
2	Boiled water extract of salmon I (2 ml. = 1.5 g.)	2.0 ml.	No	1	<25
		3.0 ml.	Yes	5	
		4.0 ml.	Yes	6	
3	Boiled water extract of salmon II (1.5 ml. = 2 g.)	1.5 ml.	No	0	<25
		4.0 ml.	Yes	10	
4	Boiled water extract of haddock	2.0 g.*	No	3	<17
		3.0 g.*	No	1	
5	Alcoholic extract of salmon (1 ml. = 1 g.)	1.0 ml.	No	2	<25
		2.0 ml.	Improved	6	
		3.0 ml.	Yes	9	
6	Alcoholic HCl extract of herring (1 ml. = 0.6 g.)	2.0 ml.	No	3	<25
		3.0 ml.	No	3	
		4.0 ml.	Yes	4	
7	Herring autolysed with toluene (putrid) (1 ml. = 2.5 g.)	0.2 ml.	No	0	65-70
		0.3 ml.	Yes	3	
		0.4 ml.	Yes	5	
8	Herring autolysed with CHCl ₃ and toluene (1 ml. = 1 g.)	0.75 ml.	No	1	25
		1.0 ml.	No	2	
		1.5 ml.	No	1	
		2.0 ml.	Yes	7	
9	Herring autolysed with toluene (1 ml. = 1 g.)	0.75 ml.	No	2	50
		1.0 ml.	Yes	7	
		1.5 ml.	Yes	7	
		2.0 ml.	Yes	6	
10	Herring digested with papain (1 ml. = 0.53 g.)	1.5 ml.	Improved	3	75
		2.0 ml.	Yes	4	
		3.0 ml.	Yes	4	
		4.0 ml.	Yes	6	
11	Wheat germ	0.2 g.	Yes	8	
12	Alcoholic HCl extract of wheat germ (1 ml. = 0.5 g.)	0.75 ml.	No	—	40
		1.0 ml.	Yes	7	
		1.0 ml.	No	2	
		1.5 ml.	Yes	5	
		1.5 ml.	Yes	2	
		2.0 ml.	Yes	3	
		2.5 ml.	Yes	3	
13	Chloroform and toluene wheat germ autolysate (1 ml. = 0.25 g.)	0.75 ml.	Yes	2	80-100
		0.75 ml.	Yes	5	
		1.0 ml.	Yes	5	
		1.5 ml.	Yes	5	
		1.5 ml.	Yes	6	
		1.5 ml.	Yes	5	
		2.0 ml.	Yes	10	

* Equivalent in fresh haddock.

Although hydrolysis with papain brought nearly all of the vitamin into solution, the method was of little use for application on a large scale for the preparation of concentrates. Our attention was therefore turned to other materials from which the vitamin might be extracted more easily. It was found

that wheat germ was exceedingly rich in vitamin B₆, containing approximately 5 units per g. (Exp. 11). We first attempted to extract the vitamin by employing the method used by Guha and Drummond [1929] for vitamin B₁, which consists of boiling the wheat germ with 50 % alcohol containing 1 % HCl, under a reflux condenser for 30 min. This method resulted in the extraction of only 40 % of the vitamin (Exp. 12). This was a better yield than was obtained from fish, but it was still thought desirable to find a method of extraction which would give almost quantitative yields. Autolysis was next tried and was found to give yields of 80–100 %. Equally good results were obtained when either a mixture of chloroform and toluene or toluene alone was employed as antiseptic. This method was finally adopted as the standard procedure for the preparation of active extracts of the vitamin.

DISCUSSION.

The results of the experiments described above may be interpreted as meaning that vitamin B₆ is largely combined in some way with the tissue in which it occurs, since the greater part is not easily extracted by ordinary solvents. In fish muscle less than 25 % of the vitamin exists in the soluble state, and more is liberated by autolysis; but for complete extraction it is necessary to digest the tissue with papain. With wheat germ, again, only 40 % is easily extracted, but all is liberated by autolysis. This behaviour is in contrast with that of the other water-soluble vitamins, such as vitamin B₁, lactoflavin and ascorbic acid, which are easily extracted, but is similar to that of factor X of Boas [1927] and vitamin H of György [1931] (the factor necessary to neutralise the toxic action of dried egg white), which is only rendered soluble by digestion with papain [György, 1935, 2]. Vitamin B₆ therefore is not properly speaking a water-soluble vitamin but occupies a position intermediate between the water-soluble group and vitamin H, which is insoluble in its natural state. No information has been obtained concerning the nature of the union between vitamin B₆ and the tissue, but possibly the vitamin is attached to the protein as a prosthetic group which is not easily split off.

EXPERIMENTAL. (For results see Table I.)

Exp. 2. 200 g. of minced salmon were boiled with water for a few minutes, then filtered, and the filtrate adjusted so that 1 ml. = 0.75 g.

Exp. 3. 200 g. of minced salmon were boiled with water for approximately 30 min., then filtered and adjusted so that 1 ml. = 1.3 g.

Exp. 4. 200 g. of minced haddock muscle were boiled with water for 30 min., filtered and adjusted so that 1 ml. = 1 g.

Exp. 5. 200 g. of minced salmon were shaken with cold 97 % alcohol, allowed to stand for a short time and then filtered. The residue was again extracted with alcohol and filtered. The combined filtrates were evaporated to remove the alcohol and made up so that 1 ml. = 1 g.

Exp. 6. 300 g. of minced fresh herring were extracted with three times their volume of 50 % alcohol containing 1 % HCl by boiling under a reflux condenser for 30 min. The alcohol was evaporated from the filtrate and the volume adjusted so that 1 ml. = 0.6 g. of fresh herring.

Exp. 7. About 7 kg. of minced herring were placed in large stone jars with twice their volume of water. Toluene was added and the jars were placed in a warm room at 20–25° for 14 days. The autolysed tissue was then filtered off and the filtrate evaporated down by boiling. A precipitate which appeared was filtered off. The volume was adjusted so that 1 ml. was equivalent to 2.5 g. of fresh herring. The autolysed tissue had a strongly putrid smell.

Exp. 8. The details of this experiment were similar to the previous one except that chloroform was added in addition to toluene. The final volume of the filtrate was adjusted so that 1 ml. = 1 g.

Exp. 9. Repetition of Exp. 7. Volume of filtrate adjusted so that 1 ml. = 1 g.

Exp. 10. 200 g. of minced fresh herring were mixed with twice their volume of water. 1 g. of a papain preparation and also a small amount of toluene were added. The mixture was incubated at 37° for 24 hours. The small amount of insoluble residue was filtered off and the filtrate adjusted so that 1 ml. = 0.53 g. of fresh herring.

Exp. 12. 200 g. of wheat germ were extracted with 1400 ml. of 50% alcohol containing 1% HCl by boiling under reflux for 30 min. The extract was filtered and the alcohol evaporated off *in vacuo*. A slight precipitate which appeared after evaporation of the alcohol was filtered off and the filtrate made up so that 1 ml. was equivalent to 0.5 g. of wheat germ.

Exp. 13. 200 g. of wheat germ were thoroughly mixed with 1400 ml. of water. Chloroform and toluene were then added, the mixture shaken for a few moments to ensure that the anti-septics were properly distributed throughout the viscous mixture. It was then incubated at 37° for 4-5 days. At the end of this period the autolysed material was filtered and the filtrate evaporated and adjusted so that 1 ml. = 0.25 g. of wheat germ.

PART II. THE CHEMICAL NATURE OF VITAMIN B₆.

The behaviour of the vitamin towards chemical reagents.

Previous to the commencement of this work little was known about the chemical behaviour of vitamin B₆, for in the earlier work on "vitamin B₂" investigators were chiefly dealing with lactoflavin. György *et al.* [1933; 1934] had noticed that their "complementary factor" which supplemented lactoflavin and vitamin B₁, and which was thought at that time to be vitamin B₄, could be adsorbed only partly on fuller's earth from impure solutions such as milk or yeast extract. These authors also found that adsorption was made more quantitative by using purified concentrates, high acidity and large amounts of fuller's earth. Later György [1934; 1935, 1] named the factor vitamin B₆ as it had markedly different properties from those ascribed by Reader to vitamin B₄, *e.g.* stability towards alkali and heat and cure of specific "acrodynia-like" dermatitis in the rat. The anti-pellagra factor for chickens of Elvehjem and Koehn [1935] is distinct from lactoflavin and has also been shown in a previous paper [Birch *et al.* 1935] to be different from vitamin B₆ and so has no bearing on the present problem. The only other work dealing with the chemical behaviour of this vitamin is that of Chick *et al.* [1935], who state that "the activity is found present in the dialysate after dialysis through a cellophane membrane and is not removed by precipitation with lead acetate either at p_H 4.0 or 8.0." We have been able to confirm these findings, as is shown in Table II, which summarises our observations. The experimental details are given in the experimental section, and the biological tests are shown in Table III.

We have not recorded the exact potencies of our most active preparations. The most active were of the order of 1 mg. and were obviously still very impure. We have been concerned in this paper with studying the chemical nature of the vitamin rather than with endeavouring to obtain preparations of a high activity. In order to do this it is of course necessary to obtain the vitamin in a moderately concentrated form. We therefore first studied the method for adsorbing the vitamin from crude extracts.

It was found that the vitamin could be adsorbed by fuller's earth from acid solution and eluted again by treatment with alkali. A fairly large amount of fuller's earth was necessary: approximately 1 g. to 10 units must be used in order to get anything approaching quantitative yields. This behaviour of the vitamin with fuller's earth is different from its behaviour with acid clay, on which it is apparently not adsorbed. Also it was noticed on one occasion that with a sample of fuller's earth obtained from Germany an active concentrate

Table II.

Reagent	Conditions	Result
Norite charcoal	p_H 6	Not adsorbed
English fuller's earth	p_H 2.5 or 5	Adsorbed, activity recovered on elution with $Ba(OH)_2$
"	p_H 9	Not adsorbed
Lead acetate	p_H 4.5 and 8	Not precipitated, activity recovered in filtrate
Silver nitrate	p_H 4 and 7	Not precipitated, activity recovered in filtrate
Mercuric nitrate	p_H 3	Not precipitated, activity recovered in filtrate
Electrodialysis in multi-compartment cell	—	Activity migrated to the cathode
Phosphotungstic acid	p_H 1	Precipitated
Picric acid	—	Not precipitated
Alcohol	Extraction of an alkaline gummy residue	Partly extracted
Acetone	Extraction of an alkaline gummy residue	Not extracted
Amyl alcohol	Extraction of an alkaline solution	Activity remained in water phase
Ether	Extraction of an alkaline solution	Activity remained in water phase
Benzoyl chloride	In alkaline solution according to the Schotten-Baumann procedure	No activity remained in filtrate
Nitrous acid	In dilute HCl	Not inactivated

Table III.

Exp.	Material tested	Amount fed ml.	Healing of "acrodynia-like" dermatitis	Weekly increase in g.	Remarks
1	Charcoal eluate (volume = 140 ml.)	0.8	No	— 3	No activity recovered
1a	Filtrate from charcoal adsorption (volume = 200 ml. approx.)	1.0 2.0	Yes Yes	7 10	About 50% of activity recovered
2	Pb acetate precipitate decomposed with H_2SO_4 (volume = 200 ml.)	0.3 0.5 0.8	No No No	— 0.5 — 0.5 2.5	No activity recovered
2a	Pb acetate filtrate decomposed with H_2S (volume = 1000 ml.)	0.5 0.75 1.0	No Yes Yes	1 5 4	Over 70% activity recovered
3	Fuller's earth eluate (volume = 200 ml.)	0.20 0.25	Yes Yes	2 2	About 25% activity recovered
3a	Fuller's earth filtrate	2.0	No	0	Over 50% activity lost
4	Fuller's earth eluate from adsorbate at p_H 2.5 (volume = 250 ml.)	0.5 2.0	Yes Yes	5 6	50% activity recovered
4a	Fuller's earth filtrate from adsorbate at p_H 2-3	1.0 2.0	No No	— 10 — 5	Over 50% activity removed
4b	Fuller's earth eluate from adsorbate at p_H 5 (volume = 250 ml.)	0.5 1.0 2.0	Yes Yes Yes	4 2 2	50% activity recovered

Table III (cont.).

Exp.	Material tested	Amount ml.	Healing of "acrodynia- like" dermatitis	Weekly increase in g.	Remarks
4c	Fuller's earth filtrate from adsorbate at p_H 5	2.0	No	0	Over 50 % activity re- moved
4d	Fuller's earth eluate from adsorbate at p_H 9	1.0 2.0	No No	Died Died	Not active
4e	Fuller's earth filtrate from adsorbate at p_H 9	1.0 2.0	Improved Yes	0 2	50 % activity remained
5	AgNO ₃ filtrate (volume = 500 ml.)	0.5 1.0	Yes Yes	2 4	Over 50 % activity re- covered
5a	AgNO ₃ precipitate at p_H 4 (volume = 250 ml.)	0.5 0.5	No No	Died Died	Not active
5b	AgNO ₃ precipitate at p_H 7 (volume = 250 ml.)	0.5 0.5	No No	Died Died	Not active
6	Hg(NO ₃) ₂ filtrate (volume = 500 ml.)	0.5 0.75 1.0	Yes Yes Yes	4 2 6	Over 75 % activity re- covered
6a	Hg(NO ₃) ₂ precipitate (volume = 250 ml.)	0.75 1.0	No No	Died Died	Not active
7	Phosphotungstic pro- cipitate (volume = 100 ml.)	0.2 0.2 0.4	Yes Improved Yes	0 Died 5	Over 50 % activity re- covered from pre- cipitate
7a	Phosphotungstic filtrate (volume = 120 ml.)	0.4	No	- 2	Less than 30 % activity in filtrate
8	Picric acid precipitate (volume = 100 ml.)	0.5	No	Died	Not active
9	Alcoholic extract of treacle (volume = 1000 ml.)	0.5 1.0 2.0	Yes Yes Yes	1 4 4	Over 50 % activity re- moved
10	Acetone extract of solution from Exp. 9 (volume = 100 ml.)	0.1	No	0	
10a	Acetone residue (volume = 500 ml.)	0.5	Yes	2	
11	Amyl alcoholic extract of alkaline treacle (volume = 100 ml.)	0.5 1.0	No No	- 9 Died - 8 Died	No activity removed
12	Ether extract of alkaline treacle	1.0	No	- 1	
13	Filtrate after benzoyl- ation	0.75 1.5 2.0	No No No	1.0 0.5 1.0	75 % activity destroyed
14	Solution after treatment with nitrous acid	0.5 1.0	Yes Yes	3 5	No loss of activity

was not obtained. However with material obtained from The British Drug Houses, Ltd., several satisfactory preparations have been made. It is possible therefore that a special type of fuller's earth is necessary in order to get proper adsorption of the vitamin. Attempts to adsorb the vitamin on norite charcoal at p_H 6 have failed, the activity always remaining in the filtrate. This result was rather surprising as it had been previously noted that the vitamin was present in the vitamin B₁ concentrates prepared according to the method of Kinnersley and Peters [1928]. However, although this method proved fruitless, we have not entirely abandoned the use of charcoal as an adsorbent; for it is well known that adsorption of a substance by this material is variable, and a slight modification of technique might result in its successful application.

The vitamin is apparently not precipitated by salts of the heavy metals such as lead, mercury or silver, but is precipitated from acid solution by phosphotungstic acid. This latter reaction suggests that it is a base, which supposition is borne out by the results of the electrodialysis experiment. It is probable however that its basic properties are not dependent on the presence of a primary amino-group for the vitamin is not inactivated by nitrous acid. On the other hand, treatment with benzoyl chloride according to the Schotten-Baumann method for the benzoylation of amino- and hydroxyl groups inactivates the vitamin. As the vitamin is not affected by nitrous acid it is possible that the destruction of the activity on benzoylation is through the involvement of a hydroxyl group.

Although the vitamin appears to be basic in nature we have not been able to precipitate it by picric acid or extract it from strongly alkaline solution by organic solvents such as ether or amyl alcohol. Its solubility in acetone has not been accurately determined, but the extraction with this solvent of a gummy residue obtained by the evaporation of an alkaline solution of the vitamin failed to bring much of the vitamin into solution, nearly all the activity remaining in the residue.

Electrodialysis experiments as a means of determining the chemical nature of the vitamin.

The object of an electrodialysis experiment is to determine whether the substance under consideration is an acid, base, ampholyte or neutral substance. If it happens to be an ampholyte an approximate measure of its isoelectric point is also obtained. This method was used by Williams and Waterman [1929] and by Birch and Guha [1931] for investigating the chemical nature of vitamin B₁ and McKinnis and King [1930] also used it for vitamin C.

Altogether three electro-dialysis experiments were carried out on vitamin B₆ (see Experimental section, p. 313). The first had to be stopped after a few hours as the concentrate used passed too high a current, but the activity was found to have migrated towards the compartment at the cathode end of the cell. In the second experiment which was carried out on a purer concentrate, electrodialysis was continued for 4 days. At the end of this period all the activity was found to have migrated to the cathode, the recovery of activity being approximately 35 %. A third experiment gave essentially similar results. It is thus evident that vitamin B₆ is either a simple base or an ampholyte having a highly alkaline isoelectric point.

Pure substances tested for vitamin B₆.

The following substances have been tested for vitamin B₆ activity but all have been found inactive in the day-dose indicated. Adenine 1 mg., adenylic acid 1 mg., yeast nucleic acid 0.2 g., choline 10 mg., betaine 10 and 50 mg. Some similarity between the chemical properties, and also the distribution in foodstuffs, of vitamin B₆ and choline made it seem possible that the two might be identical. For instance failure to precipitate the vitamin by silver or mercury excluded the purine or pyrimidine type of compound. The alkaloidal and the amino-types are excluded by failure to extract the vitamin from alkaline solution by organic solvents and by the stability of the vitamin towards nitrous acid. The curative action of fats, mention of which is made below, also indicated that choline might be concerned in some way with vitamin B₆ activity. However, pure choline chloride when fed to the deficient animals did not exert any curative action.

The sparing action of fat.

Hogan and Richardson [1934] have reported that rats fed with vitamin B which had been subjected to intense ultraviolet irradiation developed a severe dermatitis. From the description given by these authors the dermatitis appears to be similar in all respects to that produced by vitamin B₆ deficiency. In a later communication Hogan and Richardson [1935] stated that the dermatitis could be cured by wheat germ oil. This conclusion differed from our observation, for we had found that the curative factor was water-soluble rather than fat-soluble. This observation of Hogan and Richardson pointed to the supposition that two factors might be necessary for the cure of the dermatitis. However, the discrepancy between these observations has now been explained; for it has been found that fat exerts a remarkable sparing action on vitamin B₆. Thus, if a rat which has developed the dermatitis on a diet containing little fat (10% butter fat) is given small amounts of vitamin B₆ (under 1 unit) no cure is produced; if extra fat is now supplied (10 drops of linseed oil) a cure will be brought about. When a diet from which fat has been rigorously excluded is fed to rats the typical "acrodynia-like" dermatitis can be produced even while the animals are receiving relatively large amounts of vitamin B₆. This is shown in Fig. 1. By

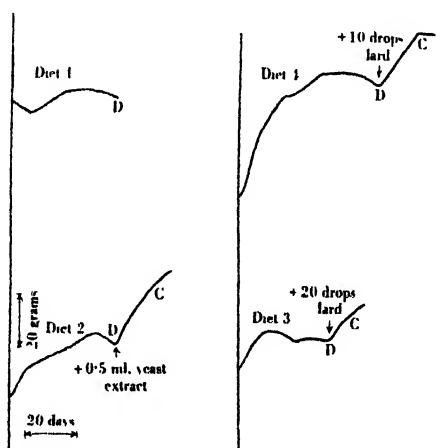


Fig. 1. Production of dermatitis in rats fed on fat-free diets containing vitamin B₆. When lard is added as indicated by arrows the dermatitis is cured and growth is resumed. Diet 1 = 20% caseinogen, 20% lard, 55% sucrose, 5% salts + 3 units vitamin B₁ and 10 γ lactoflavin daily + 1 drop halibut-liver oil per week. Diet 2 = 10% caseinogen, 9% butter fat, 66% sucrose, 4% salts, 2% agar agar, 1% C.L.O. + 3 units vitamin B₁ and 10 γ lactoflavin daily. Diet 3 = 25% caseinogen, 70% sucrose, 5% salts + 0.5 ml. alcoholic yeast extract daily + 1 drop halibut-liver oil per week. Diet 4 = 25% caseinogen, 70% sucrose, 5% salts + 1.0 ml. alcoholic yeast extract daily + 1 drop halibut-liver oil per week.

The yeast extract was made by extracting 500 g. dried yeast with 1600 ml. of 60% alcohol for 24 hours. The residue was filtered off and washed. The filtrate and washings were then evaporated to 500 ml. and extracted with ether to remove traces of fat.

supplying as little as 20 drops of lard per day to these animals growth may be restored and the symptoms cured. The possibility still remains that fats may contain appreciable amounts of vitamin B₆. However, animals develop the specific dermatitis even when receiving 30% of linseed oil or lard in the diet although the onset of the symptoms is slightly delayed and the dermatitis is not so severe.

The nature of the substance present in fats which exerts the curative action on vitamin B₆-deficient animals has not yet been determined, but results indicate that it may possibly be linoleic acid. The work of Burr and Burr [1930] has shown that linoleic acid is necessary for the normal growth of the young rat and symptoms similar to those described by these authors, *e.g.* scaly tail and scurfy appearance of the skin have often been noticed in vitamin B₆-deficient animals. In addition butter fat which has been chiefly used in diets for the production of vitamin B₆ deficiency contains only small amounts of linoleic acid, whilst linseed oil and lard which contain about 30% and 14% respectively of this substance are particularly potent in exerting a curative action on vitamin B₆-deficient animals. It is hoped to publish a further account of these observations in a later paper.

EXPERIMENTAL. (For results see Table II.)

Exp. 1. 2000 ml. of an autolysed extract of herring containing about 3 units per ml. were brought to p_H 6 and then treated with two successive lots of 25 g. of norite charcoal. Each lot was filtered off and both together were eluted with 50% alcohol adjusted to p_H 1 with HCl. The eluate was evaporated to dryness and then made up for testing.

Exp. 2. 700 ml. of an autolysed extract of herrings containing 3 units per ml. were treated with lead acetate at p_H 4.5. The precipitate was filtered off and decomposed with H₂SO₄ and H₂S. It was then made up to 200 ml. for testing. The filtrate was adjusted to p_H 8 and more lead acetate added to ensure complete precipitation; only a slight precipitate was obtained. The filtrate was then treated with H₂S to remove the lead and made up to 1 litre for testing.

Exp. 3. The filtrates from the norite charcoal adsorption and the lead acetate precipitation were combined so as to give about 4000 units in 3 litres of solution; they were then treated with two successive lots of 100 g. of English fuller's earth. The fuller's earth was filtered off, washed with water and then eluted with a mixture of pyridine 30, methyl alcohol 60, acetone 30 and water 60. The eluate was evaporated to dryness and made up to 200 ml. for testing.

Exp. 4. 3 litres of autolysed wheat germ extract, after precipitation with lead acetate, containing approximately 1 unit per ml., were divided into three equal parts. The first part was adjusted to p_H 2.5, the second to p_H 5 and the third to p_H 9. Each lot was then treated with 100 g. of English fuller's earth. The fuller's earth was filtered off and each lot eluted with the pyridine mixture given above. The eluates were evaporated to dryness and made up to 250 ml. for testing. The activity was found in the eluates from the p_H 2.5 and 5 adsorptions, but in the filtrate from the p_H 9 adsorption. Later experiments were carried out using barium hydroxide solution for eluting the fuller's earth and it was found to give as good results as did the pyridine mixture. A sample of fuller's earth of German origin was used in one experiment and no active eluate was obtained.

Exp. 5. 400 ml. of a solution obtained from a fuller's earth eluate containing 1000–2000 units were adjusted to p_H 4. Silver nitrate was added until maximum precipitation was obtained. The precipitate was filtered off and the filtrate then adjusted to p_H 7, more silver nitrate being added to ensure complete precipitation. This precipitate was also filtered off and both precipitate and filtrate were decomposed with H₂S. The precipitates were each made up to a volume of 250 ml. and the filtrate to 500 ml.

Exp. 6. Part of the filtrate from the silver precipitation was adjusted to approximately p_H 3 by means of nitric acid; mercuric nitrate dissolved in nitric acid was then added until no more precipitate appeared; the solution was then brought back to p_H 3 with NaOH and the precipitate filtered off. Mercury was removed from the precipitate by treating with H₂S, the precipitate being made up to 250 ml. and the filtrate to 500 ml.

Exp. 7. 100 ml. of a solution after precipitation with silver and mercury, containing about 700 units, were made acid to p_H 0–1 with HCl. Phosphotungstic acid dissolved in water was added until almost complete precipitation was obtained. The precipitate and filtrate were each decomposed with barium hydroxide and made up for dosing. Volume of filtrate = 120 ml., volume of precipitate = 100 ml.

Exp. 8. A solution containing 2000 units of vitamin B₆ was obtained by extracting alkaline treacle with alcohol. After evaporation of the alcohol the extract was made up to 800 ml., and 10 g. of picric acid dissolved in hot water were added. On standing in the cold, a crystalline precipitate appeared, which was filtered off and then decomposed by dissolving in warm HCl and extracting the picric acid with benzene. The HCl solution was neutralised and made up to 100 ml. for testing. The filtrate was made acid with HCl and extracted with benzene. It was found impossible to remove all the picric acid from the filtrate, and when made up for dosing the animals refused to take it properly. It was therefore impossible to say how much activity remained. A further attempt to obtain a precipitate of the vitamin with picric acid was made, using a more purified solution containing 20 units per ml. However, after addition of the picric acid no precipitate was obtained, and the solution still retained at least 50% of its activity, after the picric acid had been removed by benzene.

Exp. 9. 900 g. of black treacle, which contained about 3600 units of vitamin B₆, were made alkaline by heating on a water-bath with solid barium hydroxide. It was then transferred while hot to a Winchester bottle and 2 litres of 97% alcohol were added. The mixture was shaken by a mechanical shaker for 20 min. The alcohol extract was then poured off from the semi-solid syrup and a second extract was made. The extracts were combined and evaporated *in vacuo* to remove the alcohol and were then made up to 1 litre. Any barium present was removed with sulphuric acid.

Exp. 10. An alcoholic extract of treacle made according to the procedure given in Exp. 9 was evaporated to a sticky mass and then extracted 5 times by stirring with cold acetone. Although a considerable amount of colour was extracted all the activity was found to remain in the residue.

Exp. 11. 200 g. of treacle were diluted with an equal amount of water and made alkaline with NaOH. This solution was then extracted 5 times with amyl alcohol in a separating funnel. The amyl alcohol was evaporated off and the extracted material dissolved in 100 ml. of water for testing.

Exp. 12. The procedure given in Exp. 11 was repeated, with the use of ether instead of amyl alcohol.

Exp. 13. 500 ml. of an autolysed extract of wheat germ after precipitation with lead acetate, containing approximately 1 unit per ml., were made alkaline with NaOH and then treated with benzoyl chloride according to the Schotten-Baumann method for the benzoylation of NH₂ and OH groups. While the solution was still alkaline a large sticky precipitate appeared. On making acid, a further crystalline precipitate came down, which was also filtered off. The filtrate was then extracted with ether to remove the benzoic acid formed and was adjusted to 200 ml. for testing.

Exp. 14. A solution containing 3 units per ml., obtained from the cathode compartment of an electrodialysis of a vitamin B₆ fraction, was made acid with HCl and then treated with a 10% solution of sodium nitrite. The mixture was heated on a water-bath for 1 hour, further amounts of sodium nitrite being added until no more gas was evolved. The solution was allowed to stand overnight and the excess of nitrite was removed by means of urea. The volume was then adjusted so as to equal the original volume used.

Electrodialysis. The apparatus used by us for electrodialysis was similar to that described by Williams and Waterman [1929]. It consisted of an ebonite cell divided into 10 compartments by 9 parchment paper membranes. The 8 centre compartments held volumes of about 90 ml. each, whilst the anode and cathode held 200 ml. Platinum electrodes were used and a potential of 50 volts was generally employed.

It is necessary in performing an electrodialysis experiment of this kind to use a concentrate of the vitamin which is free from large amounts of ionisable impurities. If such impurities are present a large current is passed when the potential is applied and high concentrations of acid and alkali are formed at the anode and cathode. Under these conditions, instead of getting a gradual rise in p_H between the anode and cathode an abrupt change is obtained, which renders it impossible to determine the isoelectric point of an ampholyte with any degree of accuracy. Furthermore a considerable amount of heat is generated by a large current which, coupled with the high degree of alkalinity and acidity, is liable to inactivate the vitamin.

In the first place a preliminary experiment was carried out with a solution obtained by eluting an activated fuller's earth preparation. This concentrate passed too high a current owing to the presence of inorganic salts, and the experiment had to be stopped after a few hours. However, it was found that the activity had collected in the compartments towards the cathode end of the cell. In a later experiment an alcoholic extract of a fuller's earth eluate, taken down to dryness, was used. This preparation contained sufficient activity for the purpose and only contained small amounts of inorganic salts. The details of this experiment are as follows.

An alcoholic extract of material obtained from the elution of an activated fuller's earth preparation was evaporated to remove the alcohol and then made up to 1020 ml. This solution contained approximately 1500 units; 50 ml. were placed in both the anode and cathode compartments and 90 ml. in each of the centre compartments.

Electrodialysis was then carried out for 4 days. The current at the commencement of the experiment was 0.1 amp., but it fell considerably during the experiment until only a few milliamps were passed.

The volumes of the solutions in the various compartments and their p_H values at the end of the experiment were as follows:

Cathode 1,	202 ml.	p_H 10-12
Comp. 2,	45 ml.	p_H 7.4
" 3,	85 ml.	p_H 5.0
" 4,	73 ml.	p_H 4.5
" 5,	74 ml.	p_H 3.9
" 6, 7,	152 ml.	p_H 3.4
" 8,	77 ml.	p_H 2.4
" 9,	78 ml.	p_H 2.2
Anode 10,	134 ml.	p_H 2.0
Total	920 ml.	

On feeding the various fractions to rats it was found that the cathode solution contained approximately 3 units per ml. whilst the solutions from all other compartments were inactive in 1 ml. (See Table IV.)

Table IV.

Compartment	Amount fed	Healing of acrodynia	Weekly increase in g.
Cathode 1	0.25	Improved	1
	0.33	Yes	3
	0.50	Yes	3
" 2	0.75	No	-1
	1.0	No	0
" 3	0.5	No	-2
	1.0	No	Died
" 4	0.25	No	-4
	0.5	No	-7
" 5	2.0	No	-1
" 6, 7	1.0	No	0
" 8	1.0	No	-2
" 9	1.0	No	-2
Anode 10	1.0	No	-3
	2.0	No	-2

A second experiment was conducted in a similar manner to the one described above and again the activity was found only in the cathode.

SUMMARY.

Part I.

The quantitative extraction of vitamin B₆ from fish muscle and wheat germ has been studied. With the use of boiling water or alcohol only 25 % is extracted from fish, about 40 % from wheat germ.

On autolysis, quantitative extraction is obtained from wheat germ, but the yields from fish muscle are variable. For complete extraction from fish muscle, hydrolysis of the tissue by papain is necessary.

Part II.

An account of the behaviour of vitamin B₆ towards various chemical reagents is given.

1. The vitamin is not precipitated by salts of lead, mercury or silver, or by picric acid.

2. It is adsorbed on fuller's earth from acid solution, is precipitated by phosphotungstic acid and migrates towards the cathode on electrodialysis.

3. It is inactivated by benzoylation but not by the action of nitrous acid.

4. It is soluble in ethyl alcohol but is not extracted from a concentrated watery solution by acetone, amyl alcohol or ether.

5. From a consideration of these properties it is suggested that the vitamin does not contain a primary amino-group but is of a basic nature and possibly contains a hydroxyl group.

6. The observation by other workers that the vitamin is present in fats may be explained by our observation that fat has a sparing action on the vitamin.

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XLVIII. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES.

II. THE ALLEGED REVERSAL OF IONIC PERMEABILITY AT ALKALINE REACTION.

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UNDER physiological conditions the erythrocyte membrane is impermeable to cations. The suggestion that this impermeability might be a function of the normal ionic environment of the cells, which might be reversibly converted into a state of permeability by a suitable change in this environment, was put forward in an earlier paper [Davson, 1934]; experiments, however, failed to confirm this hypothesis in respect of Ca^{++} . The work of Mond [1927], who found that at alkaline reactions the erythrocyte became impermeable to anions and permeable to cations, together with that of Ponder and Saslow [1930; 1931] whose results pointed to a complete cationic permeability in hypertonic saline solutions seemed to provide a basis for this view.

Mond's results however are by no means above criticism. In the first place the cells were placed in isotonic sugar solution to which N NaOH had been added and the final p_H was taken as the p_H at which the change was produced. Thus 5 ml. of corpuscles were added to 6.6 ml. of a glucose solution to which 0.4 ml. of N NaOH had been added, so that in actuality the cells were added to a solution of p_H 12.8 and not to one of 10.1, the p_H at which the mixture finally arrived. To attribute any change in the membrane thus produced to a change from p_H 7.4 to one of 10.1 is quite unwarrantable. As the effect of strong bases on living tissue is particularly destructive it is only to be expected that irreversible changes in the membrane would be produced by Mond's treatment and free permeability to cations would most likely follow from these essentially irreversible changes. Mond himself remarks that he had great difficulty in preventing haemolysis by his treatment. In the second place no attempt was made to demonstrate the reversibility of the changes produced. Thirdly, the impermeability to anions at $p_H > 8.3$ was not actually proved; all that was observed was that the equilibrium ratio of Cl^- in the cells to Cl^- in the sugar solution at p_H 8.3 was not markedly different from that at $p_H > 8.3$.

In this paper it will be shown that no change in the cationic permeability occurs between p_H 7.4 and 10.0 provided that the cells are brought to these p_H values by the use of buffers; the changes in the cell Cl^- concentration observed are to be explained on the basis of the Donnan equilibrium.

EXPERIMENTAL.

The procedure already described [Davson, 1934] was employed; ox blood, which was collected directly into our own vessels from the slaughtered animal was used. The saline solutions contained Na^+ , K^+ and Ca^{++} in the proportions characteristic for ox serum and were diluted to the isotonic value with the buffer solutions. It was found necessary to wash the erythrocytes twice with the

alkaline buffer-saline solutions before they attained the desired p_H . In the first two experiments borate buffers were used but, as a certain amount of agglutination occurred under these conditions, the borate was replaced by glycine. K^+ was determined by the method of Kramer [1920] and Cl^- with a Ag-AgCl electrode. A few of the results are given in Table I.

Table I.

Exp.	Washing fluid	p_H	K+ mg./100 ml. corp.	Cl- mg./100 ml. corp.
1	Unwashed	7.4	64.8	—
	Ringer	7.4	56.0	—
	Borate/Ringer	9.0	56.0	—
2	Unwashed	7.4	67.6	—
	Ringer + KCl	7.4	88.0	—
	Borate/Ringer + KCl	8.3	88.0	—
3	Unwashed	7.4	72.7	234
	Glycine/Ringer	7.35	64.0	242
	Glycine/Ringer	9.10	65.0	153
	Glycine/Ringer	10.0	67.6	110
	Serum + Glycine/Ringer. 1 : 1	9.63	67.2	—

Results.

From Exps. 1 and 3 it is evident that the erythrocytes washed with buffer-saline solutions at p_H 8.3-10.1 lose no more K^+ than they do when washed at physiological p_H ; the loss of K^+ in the latter case has been discussed in the earlier paper and is probably due to damage of the cell membrane. It is to be noticed that serum diluted to 50% with buffer-saline at p_H 10.2 and showing a final p_H of 9.6 gives approximately the same result with regard to K^+ as ordinary buffer-saline solutions. In Exp. 2 the K^+ content of the Ringer solution was increased to 576 mg. per 100 ml. and it is seen that its penetration into the cells due probably to irreversible damage to the membrane is independent of p_H .

Returning to Exp. 3 it is seen that the Cl^- content of the cells decreases with increasing p_H ; this is to be expected on the basis of the Donnan equilibrium since the haemoglobin in the cells is ionising as an acid and this ionisation increases with the alkalinity.

DISCUSSION.

The permeability of the cell to ions has been considered to be a function of the electrical charge on its membrane [Höber, 1922]; if this were so the impermeability of the erythrocyte membrane to cations would be explained by assuming the existence of a positive charge on it. However, under the influence of an electric field the erythrocyte migrates to the positive electrode and thus there can be no doubt that its electrokinetic potential is negative. This charge is due chiefly to the layer of adsorbed proteins, since Fahreus [1921] has shown that agglutination of the erythrocytes which probably depends on their charge is dependent on the nature of the protein in the fluid in which they are suspended. The degree of agglutination is greatest in fibrinogen, less in globulin and least in albumin solutions at physiological p_H and this is the order of increasing acidity of isoelectric point. To resolve this contradiction the work of Mond has been cited [Gellhorn, 1929] as showing that another protein must exist in a certain layer of the erythrocyte membrane with an isoelectric point of p_H 8.3 which would therefore be positively charged at physiological p_H ; it is this hypothetical protein (supposed to be the globin of haemoglobin) which is said to determine the nature of the ionic permeability. Thus, if this were so, the membrane would be normally impermeable to cations and permeable to anions and

this relationship would be reversed at p_H 8.3. In actual fact the presence of free globin has never been established in the erythrocyte membrane and the theory is based on experimental facts which this paper has shown to be quite unreliable and, as will be shown later, on a misconception of the nature of the potentials which determine ionic penetration.

In the interpretation of the influence of the potential of a membrane on its permeability properties care has to be taken to distinguish between the electrokinetic ζ - and ϵ -potentials. The ϵ -potential across an interface is determined partly by the asymmetrical distribution of ions in the neighbourhood of the interface and partly by the non-ionic electrical dipoles of the molecules oriented at the interface. Of these two components only one, the ionic term, changes its sign at the isoelectric point; the other, the dipole term, has a sign and magnitude which are largely independent of p_H changes. The dipoles are definitely fixed at the interface, while the ions form a rather diffuse layer. Some of these ions are free to move independently of the interface, others are not. Thus when an electric field is applied to this system the two sets of ions will move separately. The potential difference between the set of ions fixed in the surface and the set in the bulk phase is known as the electrokinetic ζ -potential. The electrokinetic potential being dependent on the existence of ions changes its sign at the isoelectric point where it is zero. It will thus be clear that, since ϵ is due to the sum of two terms (dipole moment and lack of symmetry in ionic distribution) and ζ is due to another term, namely the independence of motion of some of the ions, and is not directly related to either of these other two, ϵ and ζ will not be

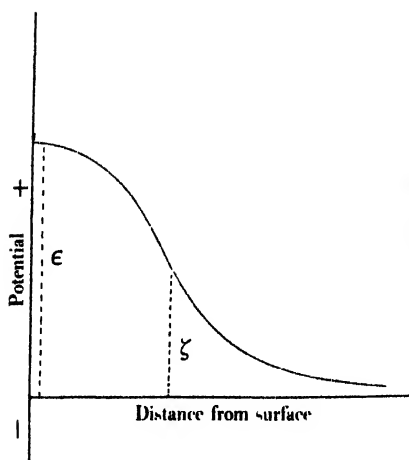


Fig. 1.

Fig. 1. Difference of ϵ - and ζ -potentials.

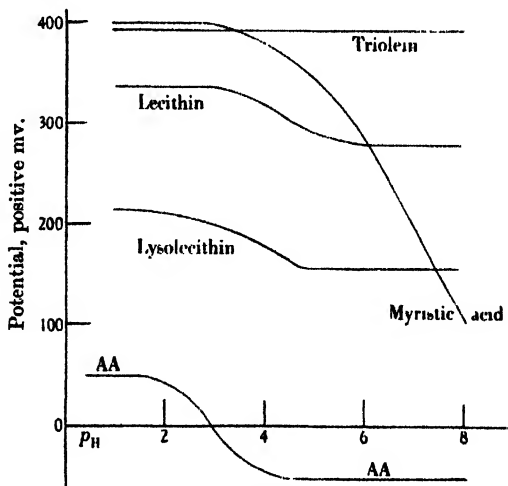


Fig. 2.

Fig. 2. ϵ - and ζ -potentials. Curve AA illustrates the potential changes with p_H for lecithin. The remaining curves are for ϵ , taken from Hughes [1935].

identical and may even be of different signs. This is illustrated diagrammatically in Fig. 1 and in Fig. 2 are shown ϵ and ζ curves for lecithin. It is evident from the figure that whilst ζ changes its sign at the isoelectric point, ϵ remains positive over the whole range of p_H investigated. In fact all the substances which are supposed to be in the erythrocyte membrane have positive ϵ -potentials at all p_H values, so far as has been investigated.

With regard to the penetration of ions through a membrane, there are three suggested mechanisms for the process.

(1) If the mechanism is that of the passage through a homogeneous lipid layer, and not through a pore, then ϵ is the potential of interest. Since in the case of the erythrocyte ϵ is probably always positive and is not changed in sign by p_{H} changes, a reversal of ionic permeability with change of p_{H} is not to be expected.

(2) If penetration is through a pore in the membrane, as suggested by Michaelis [1925], ζ is the potential of interest. That this is so will be obvious from Fig. 3a. The ions which can penetrate through such a pore must be (a) free to

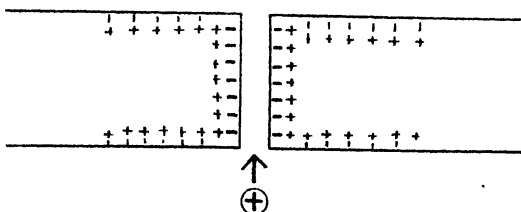


Fig. 3a. Pore mechanism of ionic penetration.

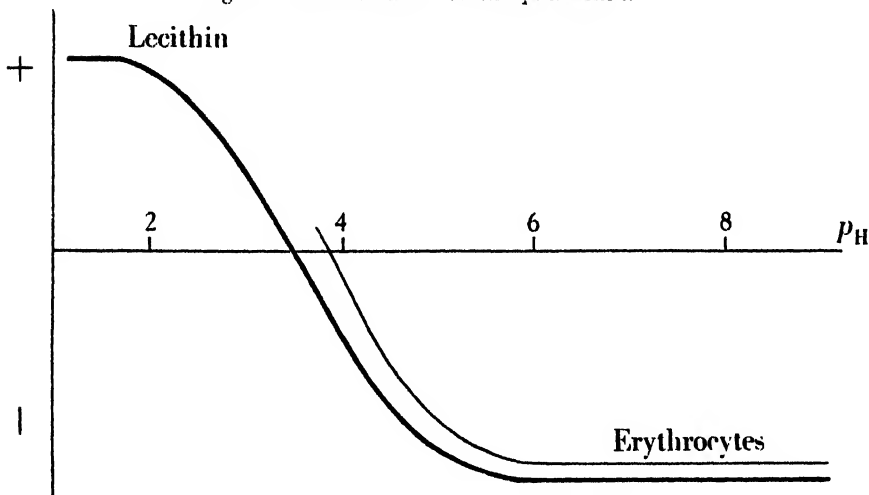


Fig. 3b. ζ - p_{H} curves for lecithin and erythrocytes.

move and (b) of opposite sign to the fixed ions of the pore walls. Hence when the walls ionise as an acid cations will penetrate, and when they ionise as a base anions will penetrate. Thus there should be a reversal of ionic penetration at the isoelectric point of the erythrocyte wall. The work of Coulter [1920], *e.g.*, shows that the isoelectric point of the erythrocyte is at about p_{H} 4.75 as is shown in Fig. 3b. Hence over the range of p_{H} 7–10 the pore surface should ionise as an acid and cations only should penetrate, which is not so.

(3) If the ions penetrate by combination with a component of the erythrocyte membrane followed by diffusion through the membrane as an ionic doublet, as suggested by Osterhout [1930], neither ϵ nor ζ is of direct importance. However, if a cation is to penetrate, the surface molecules must ionise as an acid. Thus only cations will be able to penetrate on the alkaline side of the isoelectric point and only anions on the acid side. Thus again there should be a reversal of the sign of ionic penetration at p_{H} 4.75.

In the present paper it has been shown that over the range of p_H 7–10 anions only penetrate the erythrocyte wall. Hence mechanism (1) is the only possible one for the erythrocyte. From the above considerations we are led to a picture of penetration through the erythrocyte membrane in which the potentials encountered by ions are as shown in Fig. 4. The form of the potential curve

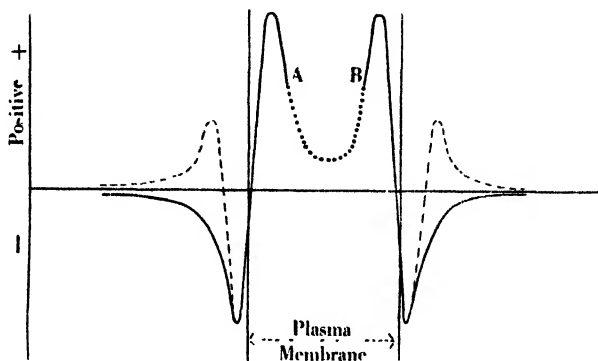


Fig. 4. Potential diagram perpendicular to the plane of thin lipid membrane. Continuous line when ionising as a base; broken line when ionising as an acid. Dotted portion: potential undefined.

between *A* and *B* is difficult to define but is not of significance for this argument. The important point is that the plasma membrane has an excess of positive charge due to the oriented dipoles of the interfaces and hence the solubility of anions in the membrane will be much greater than that of cations. The establishment of equilibrium by ionic exchange should therefore be rapid in the case of anions and slow in that of cations, possibly of negligible speed as found here experimentally.

SUMMARY.

1. The work of Mond on the change in the ionic permeability of erythrocytes at p_H 8.3 is criticised. Using buffer solutions to bring the erythrocyte to p_H 8–10 no reversal of ionic permeability was obtained.
2. The potentials at the membrane interfaces are discussed in relation to theories of the mechanism of ionic penetration.

Our thanks are due to Prof. J. C. Drummond for his kind interest in this work, to the Medical Research Council for defraying the expenses of the research and for a personal grant to one of us (H. D.), and to the Department of Scientific and Industrial Research for a personal grant to J. F. D.

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XLIX. A CONSTANT-VOLUME APPARATUS FOR GAS ANALYSIS.

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(Received October 12th, 1935.)

IN the course of investigations on the chemistry of respiration in fruits a constant-volume gas analysis apparatus (Fig. 1) has been developed in this laboratory. A 5 ml. sample of the respired air is taken into the burette (*B*) and is passed into one of the absorption bulbs (*C*, *D*). When one of the components has been completely absorbed it is brought back into the burette and the decrease in pressure noted, the volume being kept constant. This decrease in pressure represents the partial pressure of the component under consideration and is proportional to its concentration in the gaseous mixture. If *h* is the decrease in pressure and *H*₀ represents the atmospheric pressure, the percentage content of the constituent, say *x*, is easily computed: $x = h \times 100/H_0$.

The apparatus (Fig. 1) consists of a manometer (constructed out of 3 mm. bore glass tubing) one limb of which carries a 200 mm. scale etched on it whilst the other is blown into a pipette (*B*), the volume of which between the tap (*G*) and the circular mark (*X*) is exactly 5 ml. Within the water-jacket is also placed a thermo-barometer [Warburg, 1926] which is similar in construction to the manometer just described, with the difference that mercury level adjustments are made by means of the screw (*S*) which presses a rubber reservoir (*R*). One of the limbs of the thermo-barometer carries a 10 mm. scale etched on either side of the zero mark, which corresponds with the mark (*X'*) on the pipette (*B'*). The bulb (*D*) is similar to the "combustion pipette" employed by Haldane [1912] except that the ignition tubes inside the pipette have been removed and a water levelling bulb added. The bulb (*D*) contains stick yellow phosphorus, kept under water, for the absorption of oxygen [Carpenter, 1915]. The KOH bulb (*C*) also resembles the one employed by Haldane [1912], and is filled with a 30 % solution of KOH for CO₂ absorption. A compensation pipette (*H*), based on the same principle as the one devised by Haldane [1912], is added to the KOH bulb.

Sampling is done by the washing method [Carpenter, 1915] by interposing a 3-way tap between the inlet of the pipette (*B*) and the respiration chamber. After the final washing is completed exactly 5 ml. of the sample are taken into the pipette (*B*), the mercury level being adjusted to the mark (*X*) by means of the levelling bulb (*L*). The tap (*G'*) of the thermo-barometer is momentarily opened and is immediately closed after adjusting the mercury to the zero mark on the scale and the circular mark (*X'*) on the pipette (*B'*), the adjustment being made by means of the screw (*S*). The stopcock (*F*) is turned in the position (*Fa*), the tap (*E*) kept in the position shown in Fig. 1, the tap (*K*) opened and the level of KOH adjusted to the circular marks on the compensation pipette and the KOH bulb. The 3-way tap (*F*) is now turned in the position (*Fb*), the tap (*K*) closed and the tap (*G*) turned so that the gas pipette (*B*) communicates with the KOH bulb. The gas is then driven into the KOH bulb and passed backwards and forwards several times. After finally bringing back the gas into the pipette

(B), the potash levels are set and the tap (G) half-turned, so that the gas is confined in the pipette (B). The mercury is again brought to the mark (X) and the lowering of pressure read. The reading of the thermo-barometer is added to or

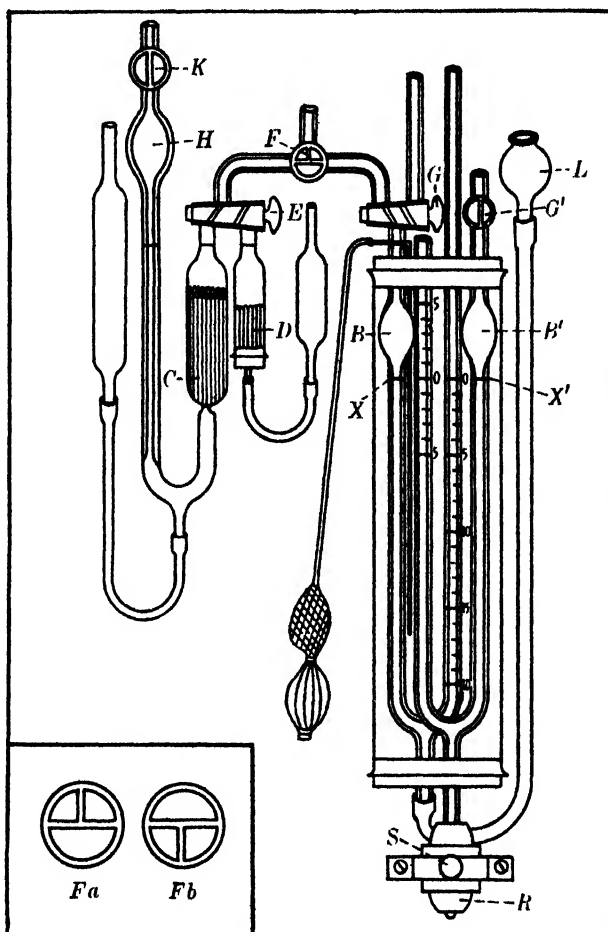


Fig. 1.

subtracted from that of the manometer in order to eliminate errors due to variations in external conditions. The oxygen is absorbed by a similar process except that the gaseous sample need not be continuously agitated.

The accuracy of the apparatus, as found in practice by a series of analyses of outdoor air, is $\pm 0.1\%$.

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L. A MANOMETER FOR COMPARATIVE STUDY OF PHYSIOLOGICAL PROCESSES.

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(Received December 29th, 1935.)

IN the course of investigations on the purification of the polyphenol oxidase of potato it was found necessary to measure the degree to which purification had been achieved in the various enzyme preparations. The degree of purification of an enzyme is generally denoted by a number which is the ratio of enzymic activity per mg. dry weight of final preparation to the enzymic activity per mg. dry weight of the original material [Haldane, 1930]. Warburg and Barcroft manometers are inconvenient for the purpose, as what is required is the ratio and not the actual amounts of the oxygen absorbed by the final preparation and the material from which it has been obtained. It was with the idea of developing an apparatus in which the scale readings could be directly compared in order to get the degree of purification of an enzyme, and which could, at the same time, be employed for quantitative determinations, that the manometer described in the present note has been constructed.

It is arranged that both the vessels (*P*) and (*Q*) of the apparatus (Fig. 1) shall have the same vessel-constant; methods of ensuring this have been described in detail by Dickens and Greville [1933]. The gas in the pipette (*C*) is maintained at the initial atmospheric pressure throughout, the tap (*E*) being closed after the Brodie solution has been adjusted to the mark (*X*) at the commencement of the experiment. When it is required to compare two reaction velocities, involving the evolution or absorption of the same gas, all that is necessary is to compare the scale readings in the two graduated limbs (*A*) and (*B*). The apparatus can also be used for quantitative determinations, in which case one of the vessels is employed for the main reaction while the other serves to indicate the fluctuations in the temperature of the water-bath. If the vertical distance between the menisci in the two manometric limbs (*A*) and (*B*) be *h*, and *x* the amount of gas evolved in μ l. at N.T.P. (dry), we have

$$x = h \cdot k'_v.$$

The calibration of the apparatus consists in finding the value of this constant k'_v , which is calculated from the following simplified formula¹:

$$k'_v = \frac{Vg \frac{273}{t+273} + Vf \cdot \alpha'_v}{P_0} + A \frac{273}{t+273},$$

where $Vg = \mu$ l. gas in reaction vessel and manometer tubing down to the mid-point on the scale; $Vf = \mu$ l. liquid in reaction vessel; α'_v = Bunsen absorption coefficient of the gas at t° ; P_0 = pressure of one atmosphere expressed in mm. of manometer liquid; A = area of cross-section of the manometer tube in mm.²

¹ It may be noted that in this formula the coefficient of A is unity and not $\frac{1}{2}$ as in the differential manometer, the reason being that in this case the depression of liquid in one graduated limb is not accompanied by a corresponding rise in the other.

The manometric limbs (*A*) and (*B*) and the central pipette (*C*) are constructed out of glass tubing with an internal cross-sectional area not exceeding $\frac{1}{2}$ mm.² The central pipette (*C*) carries a circular mark (*X*) exactly corresponding with the 150 mm. marks on either side-limb, beyond which it is blown into a small bulb (*D*), capacity about 3.5 ml., and carries a tap (*E*) at its extremity. The two side-limbs carry mm. scales, 0 to 300, etched on them. To maintain it at a uniform temperature during experimentation, the bulb (*D*) is covered by a piece

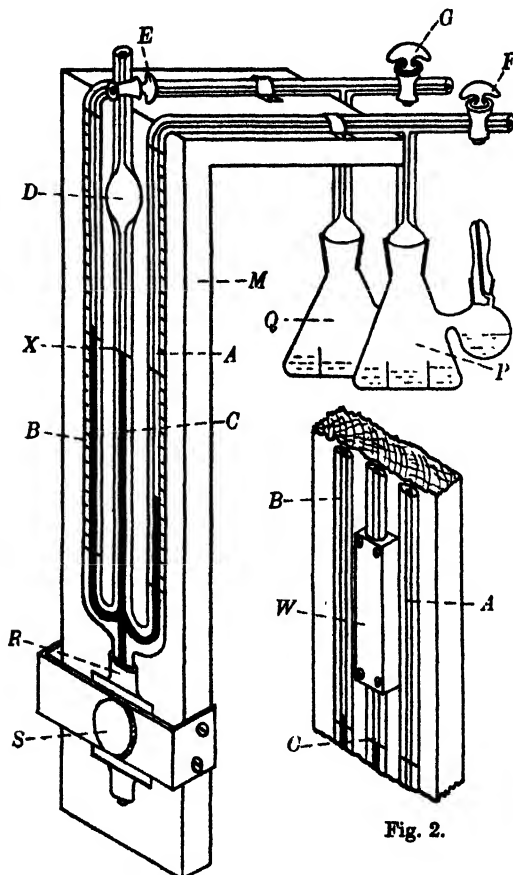


Fig. 1.

of flannel, and a rectangular piece of wood (*W*), hollowed on one side so as to fit on the bulb, is screwed on to the wooden board (*M*) carrying the apparatus. Fig. 2 depicts a portion of the apparatus with the wooden piece (*W*) in position. A screw-clamp arrangement with a rubber reservoir (*R*) is provided, the manometric liquid being adjusted by means of the screw (*S*). The taps (*F*) and (*G*) are of the type used by Dixon and Elliott [1930] with the Barcroft respirometer. The vessels used are those generally employed with Warburg manometers. Each vessel possesses a side-bulb, above which an outlet is provided with a stopper of the type introduced by Warburg and Kubowitz [1929] which is so constructed as to act also as a tap and the outlet may be closed by giving it a half turn. The

vessels should be made nearly the same size; several methods [Dickens and Greville, 1933] are then available for securing equality of volumes.¹

The manipulation is easy. By means of the screw (*S*) the manometric liquid is adjusted to the mark (*X*) on the pipette (*C*) when it should also stand at the 150 mm. marks on both the side-limbs. During this adjustment all the three limbs (*A*), (*B*) and (*C*) are kept in communication with the air, the taps (*E*), (*F*) and (*G*) being subsequently closed. At the conclusion of the experiment, the Brodie solution is again adjusted to the mark (*X*) on the pipette (*C*) and the scale readings in the two graduated limbs are read and directly compared.

When used for quantitative determinations the accuracy of the apparatus, as found by liberating known amounts of CO₂ by adding measured amounts of standard acid from the side-bulbs to an excess of the bicarbonate solution contained in the main part of the vessels, is $\pm 1.25\%$.

¹ Equality of vessels is easily checked by the following method. Empty vessels are worked on to their ground joints and secured by rubber bands. After the manometric liquid has been brought to the mid-points in all the three limbs, the taps are closed as usual. Now by means of the screw (*S*) the manometric liquid is adjusted to a point much higher than the point (*X*) on the central pipette. Any difference of reading on the two side-limbs indicates an inequality of vessels and is corrected by introducing a small glass bead or by blowing the side-bulb of one of the vessels.

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LI. SOME PROPERTIES OF THE REDUCING MATERIAL IN CERTAIN FRACTIONS OF NORMAL URINES.

I. THE NATURE OF THE "FREE" FERMENTABLE SUGARS AND THE FERMENTABLE SUGARS PRODUCED ON HYDROLYSIS IN "FASTING" URINES.

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(Received December 23rd, 1935.)

THE problem of the occurrence of glucose in "fasting" urine was investigated recently as far as available methods would permit by Harding and Selby [1931; 1933] and by West *et al.* [1932]. Both agreed that human "fasting" urine contained small amounts of reducing material removable by baker's yeast. West and Steiner [1932] differed from Harding and Selby in believing that the fermentable substance was all glucose. Recently Harding and Nicholson [1933] developed a method for quantitative determination of small amounts of sugars in solution by specific fermentation. It was thought that the use of this method, which for brevity has been called "mycological" (although the term is not strictly applicable), might throw further light on the nature of free "fermentable" sugar in fasting urine and also on the nature of the "fermentable" sugar produced on hydrolysis.

West *et al.* [1932] have emphasised the prime importance of separating non-carbohydrate reducing substances of urine as completely as possible from the carbohydrates; by using $\text{HgSO}_4\text{-BaCO}_3$ they obtained filtrates almost entirely free from nitrogenous interfering substances. The mycological method of sugar analysis was applied to urines treated in this way but the filtrates contained nitrates which interfered with the estimation of glucose by *Proteus vulgaris*.¹ Other unidentified substances which interfered with the mycological method of analysis when applied to concentrated filtrates were found in some urines. For these reasons a further fractionation of the urine was necessary. By applying a modification of the copper-lime method [Salkowski, 1879; Van Slyke, 1917] to urines cleared with basic lead acetate followed by $\text{HgSO}_4\text{-BaCO}_3$ [West *et al.*, 1929], solutions were obtained which gave satisfactory results.

¹ The action of *P. vulgaris* converted the nitrates into nitrites. The latter would later oxidise the KI used with the Harding-Downs reagent, liberating free iodine and thus giving reducing values which were much too low.

Analytical methods.

Reagents: Basic lead acetate solution [Plimmer, 1915].

Mercuric sulphate [West *et al.*, 1929].

Barium carbonate.¹

Proteus vulgaris

Monilia krusei

Monilia tropicalis

[Harding and Nicholson, 1933].

Saccharomyces marxianus [Harding *et al.*, 1933].

Sugar reagent—modified Shaffer-Hartmann [Harding and Downs, 1933].

20% Copper sulphate solution.

8% Calcium hydroxide suspension.

All determinations of reducing material were done by the method of Harding and Downs [1933] with the modification that the time of boiling was increased to 20 min. in accordance with the recommendation of Shaffer and Somogyi [1933] for similar copper solutions. In order to get a sufficient amount of solution and to obtain "average" results "composite" fasting urine specimens were used. The urine excreted by six normal individuals between 8 a.m. and 12 noon (the last food having been taken at 6 p.m. the previous day) was mixed and made up to 1500 ml. with distilled water.

Preliminary clearing.

To this solution 185 ml. of basic lead acetate solution [Plimmer, 1915] were added and the volume made up to 3000 ml. After standing for half an hour with occasional shaking the precipitate was removed by centrifuging. Excess Pb was removed by precipitation with H_2S and filtration. Excess H_2S was then removed by aeration.² To every 250 ml. of this filtrate 100 ml. of $HgSO_4$ reagent [West *et al.*, 1929] were added. Distilled water was added to make the final volume twice that of the basic lead acetate filtrate used. Powdered $BaCO_3$ was added with constant shaking until the mixture was slightly alkaline. The precipitate was removed by suction filtration. The filtrate was made distinctly acid with concentrated H_2SO_4 and the Hg remaining in solution³ was removed by precipitation with H_2S .⁴ After suction filtration the excess H_2S was removed by aeration

¹ The barium carbonate was prepared either by passing a stream of CO_2 through a hot concentrated $Ba(OH)_2$ solution or by adding a hot solution of Na_2CO_3 to a hot solution of $BaCl_2$. After washing and drying the $BaCO_3$ was finely pulverised by passing through the finest mesh grinder of a meat chopper.

² The conditions for obtaining the optimum "clearing" of urines with basic lead acetate, without loss of fermentable sugar, were determined by Mr S. H. Jackson working in this laboratory. A more complete discussion of the method is given in the second paper of this series [Harding, Nicholson and Jackson, 1936].

³ Owing to the presence of urea, this filtrate contained a much greater quantity of mercuric salt than would be present if urea were absent. This indicates that urea unites with Hg^{++} to form a compound which is moderately soluble in alkaline solution. Supposedly, Hg^{++} is in equilibrium with the almost insoluble $HgCO_3$ and the urea complex which is only slightly dissociated in mildly alkaline solutions. This explains why, after the use of a certain quantity of $HgSO_4$ reagent, the use of additional amounts fails to remove appreciably greater quantities of urea. The reducing power of the filtrate is even less affected than the nitrogen content by the use of increased amounts of $HgSO_4$.

⁴ West *et al.* [1929] recommend the use of H_2S instead of zinc for the removal of $HgSO_4$ because the use of zinc would result in the production of H_2O_2 . We have found that another disadvantage in the use of zinc is that it reduces nitrate to nitrite which interferes with the determination of sugar in the manner described in the footnote on page 326.

(Filtrate = Fraction A). The filtrate (about 3945 ml.) was adjusted to p_H 6.5 and concentrated by evaporation under reduced pressure at 37° to a final volume of 150 ml.

Copper-lime procedure.

The concentrated filtrate was chilled to 0° in a large centrifuge bottle. 4 ml. of 20% $CuSO_4$ and 8 ml. of 8% fine suspension of $Ca(OH)_2$ were added and stirred intermittently (at 0°) for 30 min. The copper-lime precipitate was then centrifuged at about 2000 r.p.m. The supernatant fluid was poured off and termed the copper-lime filtrate. The inside of the bottle was rinsed and the precipitate thoroughly mixed with four successive portions of 5 ml. of cold saturated $Ca(OH)_2$ solution. After the addition of each 5 ml. portion the precipitate was centrifuged and the washings added to the filtrate. After the fourth washing the precipitate was suspended in 50 ml. of water and the sugar regenerated by acidification with a slight excess of concentrated H_2SO_4 .

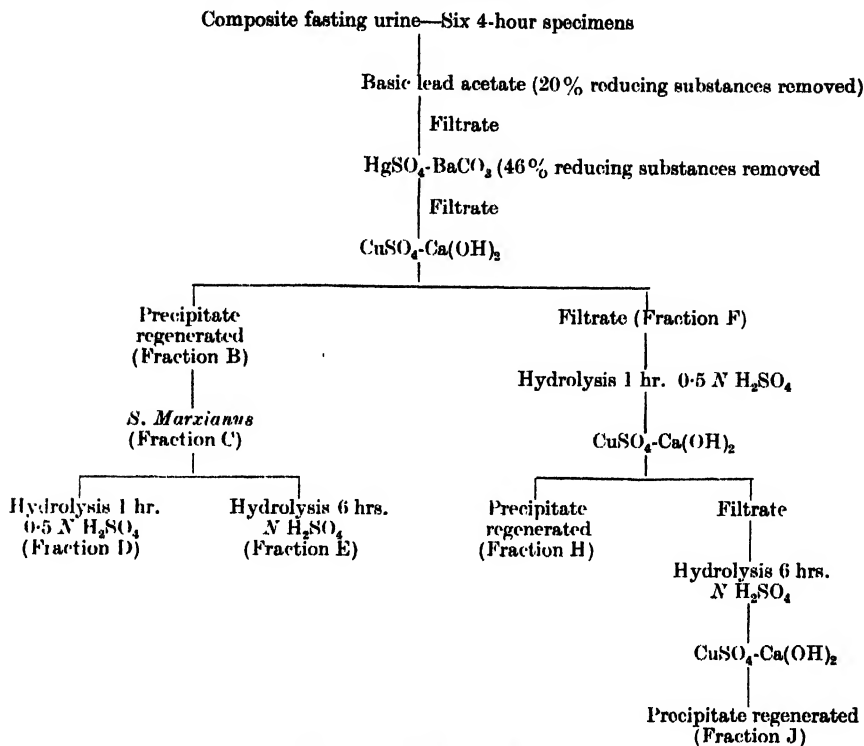
The mixture of copper-lime filtrate and washings was then treated again under the same conditions with the same quantities of $CuSO_4$ and $Ca(OH)_2$. This procedure was repeated until four precipitations had been effected. Each precipitate was washed four times as above. The four regenerated precipitates were combined. Cu^{++} was removed by precipitation as CuS with H_2S . After filtering, the H_2S was removed by aeration, Ca^{++} with KH_2PO_4 and MgO by the same technique as was used in removal of NH_4^+ [Harding and Downs, 1933]. The resulting solution was called the first copper-lime precipitate or Fraction B and contained all the free fermentable material. The filtrate was acidified and cleared of Cu^{++} and Ca^{++} by the same methods. This was the first copper-lime filtrate or Fraction F. Water was added to both fractions to make them up to known suitable dilutions.

A portion of Fraction B was removed for differential sugar analysis by the method of Harding and Nicholson [1933]. The remainder was incubated for 30 min. at 37° with *S. marxianus* (0.5 g. packed wet weight per 10 ml. of solution) to remove the free sugar including galactose. The yeast was removed by centrifuging and the supernatant solution (Fraction C) divided into two portions, one of which was hydrolysed by boiling with 0.5 *N* H_2SO_4 for one hour under a reflux condenser (Fraction D). The other portion was hydrolysed by boiling with 0.1 *N* H_2SO_4 for 6 hours under similar conditions (Fraction E). After neutralising the hydrolysate with $BaCO_3$ the sugar produced by hydrolysis was analysed by the mycological method. This "fermentable" sugar produced on hydrolysis will for convenience be referred to as the "hydrolysis sugar".

In two instances Fraction C was hydrolysed continuously for 6 hours with *N* H_2SO_4 , small portions being removed at intervals for differential sugar analysis. The first copper-lime filtrate (Fraction F) was hydrolysed by boiling for 1 hour with 0.5 *N* H_2SO_4 , neutralised, the ammonia produced by hydrolysis of urea removed by the use of KH_2PO_4 and MgO and the free sugar produced removed by the copper-lime method. The sugar precipitated (Fraction H) was analysed as before. The filtrate from this precipitation was hydrolysed further by boiling with *N* H_2SO_4 for 6 hours, neutralised, cleared of ammonia and the free sugars precipitated with copper-lime. The recovered sugars (Fraction J) were analysed mycologically.

Before treatment with the micro-organism all solutions were adjusted to p_H 6.5 (approx.) [Harding and Nicholson, 1933].

The various steps in the fractionation procedure are illustrated in the diagram.



Discussion of methods.

The determination of fermentable sugar in normal fasting urine by the use of ordinary yeast can be made quite satisfactorily on Lloyd's or HgSO₄-BaCO₃ filtrates when the urine is diluted 1:7 or more. At these concentrations the mycological method of differential sugar analysis can be employed if the filtrate is treated with H₂S after the action of *P. vulgaris* to reduce the nitrites formed (see footnote, page 326) to ammonia. At these concentrations however it is impossible to say with certainty whether any sugar, other than glucose, is present. In some specimens galactose and in a small number fructose appears to exist, but the amounts found are so close to the limits of error of the methods of analysis used that it is evident that a greater concentration of the urine filtrate is necessary. This was accomplished by vacuum evaporation. No phosphate was added and the *p_H* remained constant. West and Steiner [1932] noted a considerable loss of sugar during vacuum evaporation of HgSO₄-BaCO₃ urine filtrates in the presence of added phosphate. Under our conditions no loss of "fermentable" or "non-fermentable" reducing material was noted during the process and no reducing material was found in the distillate. The concentrates were diluted to give a dilution of from 1:1.5 to 1:3 on the original urine. At these concentrations the amount of the non-fermentable reducing substances was so high that it almost exceeded the limits of accuracy of the copper reagent used and all the yeasts, including baker's yeast, showed a continuous removal on repeated fermentations similar to that observed by Van Slyke and Hawkins [1929] on unclarified urines. The urea concentration is also quite high, producing much ammonia on hydrolysis. The substance giving the secondary yeast removal could be taken out of solution by preliminary treatment with basic lead

acetate, which also decreased the non-fermentable reducing substances and organic nitrogen to a slight extent (see footnote, p. 326), but the amounts of non-fermentable reducing substances and of urea in the concentrated filtrates were still too great. Another difficulty was the presence of a considerable amount of nitrate which interfered with the use of *P. vulgaris* for the estimation of glucose (see footnote, p. 326). The use of the modified copper-lime procedure overcame these difficulties. By this method it was possible to remove all the "free" fermentable sugar from urine with a maximum error of 3%. Added sugars were recovered with the same degree of accuracy. No appreciable Lobry de Bruyn conversion occurred, the added sugars being recovered unchanged and identified by hypiodite titrations and the mycological method of differential analysis [Archibald, 1935]. The regenerated precipitates were free from nitrates and organic nitrogen. Some of the substance giving the secondary yeast removals was precipitated by the copper-lime method from the $\text{HgSO}_4\text{-BaCO}_3$ filtrates, necessitating the use of the preliminary treatment with lead acetate. By the procedure used, quantitative determinations of the "free" fermentable sugars and the "hydrolysis" sugars in both the copper-lime precipitate and copper-lime filtrate portions of the lead acetate- $\text{HgSO}_4\text{-BaCO}_3$ urine filtrates could be made. These represented the total "free" fermentable and "hydrolysis" sugar present in such urine filtrates. No sugars were considered to be present unless they were in the urine fractions in concentrations sufficient to give a reduction equivalent to 0.50 mg./100 ml. glucose (a titration figure of 0.08 ml. of 0.005 *N* $\text{Na}_2\text{S}_2\text{O}_5$). The dilutions of the different fractions varied from 3.0 to 0.5. Titrations were done in triplicate and usually agreed within 0.02 ml.

The use of the two strengths of acid and the two periods of hydrolysis was found advisable because, although sucrose, maltose and lactose were hydrolysed completely by refluxing for 1 hour with 0.5 *N* H_2SO_4 , the urine filtrate contained some substance which was very slowly hydrolysed. The milder treatment was used first because of the greater destructive action of the more prolonged hydrolysis with the stronger acid on some of the reducing substance found in urine. The 6-hour refluxing of urine filtrates with normal acid caused some destruction of both fermentable and non-fermentable carbohydrate and the production of some furfuraldehyde and charring. Ordinary sugars under similar conditions did not char. The charring was probably the result of reaction of urine phenols with furfuraldehyde liberated by acid destruction of urine sugar. The charring and furfuraldehyde production on hydrolysis were not nearly as marked in either of the copper-lime fractions as in Fraction A, probably owing to the fact that most of the furfuraldehyde-producing compounds were in Fraction B whilst the phenols were largely in the copper-lime filtrate. Although Everett *et al.* [1927] showed that appreciable amounts of fructose were destroyed during acid hydrolysis, no destruction of fructose occurred under the conditions of milder hydrolysis used here. Considerable amounts of fructose were destroyed by the more prolonged hydrolysis. Glucose was affected very little by either procedure. Harding and Grant [1931] demonstrated that prolonged hydrolysis of lactose resulted in an alteration of the ratio of measurable glucose and galactose. After 6 hours' hydrolysis of lactose with *N* H_2SO_4 , analysis with *M. tropicalis* and *M. krusei* showed theoretical values for glucose and a slightly raised figure for galactose.

Neutralisation with a salt which forms an almost insoluble sulphate was necessary. Otherwise the salt concentration (7% Na_2SO_4 if NaOH be used to neutralise *N* H_2SO_4) was sufficient to inhibit completely the action of *P. vulgaris*. Partial inhibition of the action of *P. vulgaris* occurs in solutions containing 3% or

more of sodium salt. Such salt concentrations also increase the reducing value of the non-fermentable fractions to a very considerable extent (10–15%), although they have no effect on the fermentable fraction.

It has been shown that yeast juice acting on sugar produces a polysaccharide [Cremer, 1899; Harden and Young, 1913; Naganishi, 1926]. It was thought possible that some of the "hydrolysis" sugars might have been produced in this way by the action of the organism (*M. tropicalis* or *S. marxianus*) used to remove the "fermentable" sugars from the solution before hydrolysis. That this did not occur however was shown by the fact that hydrolysis of blanks consisting of fermented glucose solution of about the same concentration as the fermentable sugars in the urine fractions gave no reducing substance on hydrolysis, and the "hydrolysis" sugars in the fermented urine fractions did not differ appreciably from the "hydrolysis" sugar calculated by subtracting the fermentable sugars found in the unhydrolysed urine fraction from the total "fermentable" sugars found after hydrolysis of the "unfermented" fraction.

The "free" fermentable sugar and fermentable sugar produced on hydrolysis in fasting human urine.

Since the micro-organisms were used only on urines which had undergone some clearing, those carbohydrates or carbohydrate derivatives which were completely precipitated by the clearing reagent lay entirely outside the scope of this study. In the case of those substances which are only partly precipitated by the clearing reagent, the amounts reported present in the filtrates do not represent the amounts present in whole urine. The reducing fraction lost in clearing would include the oxypoteic fraction examined by Gwozdz [1933].

Typical results obtained by the differential sugar analysis of the various urine fractions are shown in Tables I and II and in Fig. 1. The results are expressed as mg. per 4 hours per single urine specimen. For simplicity in calculating

Table I. *Sugars of composite fasting male urines as mg. of glucose per 4 hours.*

	Fraction B	Fraction D	Fraction E	Fraction H	Fraction J
Total sugar	28.5	25.9	26.8	2.7	4.8
Glucose	6.0	4.7	7.0	0.5	1.6
Fructose and/or mannose	1.6	0.9	0.3	—	—
Galactose	1.7	3.2	3.6	0.2	0.3
Non-fermentable	19.2	17.1	15.9	2.0	2.9

Table II. *Sugars of composite fasting female urines as mg. of glucose per 4 hours.*

	Fraction B	Fraction D	Fraction E	Fraction H	Fraction J
Total sugar	19.2	22.0	23.8	2.4	4.0
Glucose	4.4	3.6*	7.5*	0.3	2.1
Fructose and/or mannose	0.0	—	—	—	—
Galactose	1.5	1.1	3.6	0.3	0.0
Non-fermentable	13.3	17.3*	12.6*	1.7	1.9

* The determinations of "hydrolysis" sugars of female urines were done before the effects of salt concentration on the action of *P. vulgaris* and on the reducing power of the non-fermentable fraction were observed. No differentiation of glucose from fructose and/or mannose was possible and the non-fermentable fractions were presumably 10–15% too high.

"non-fermentable" by subtracting "total fermentable" from "total" sugar the amounts of all sugars are expressed in terms of glucose. Glucose accounts for by far the largest portion of the "free" fermentable reducing substance present in

urine filtrates.¹ Very small amounts of fructose and/or mannose are found in some but not all urines. Galactose occurs in appreciable amounts in some urines, being proportionately higher in female urines. This sugar however would not be included in the fermentable sugar reported by other workers since baker's and brewer's yeasts do not remove galactose. After hydrolysis of the fermented first copper-lime precipitate (Fraction D) considerable amounts of galactose were found. The glucose found after the prolonged hydrolysis was always considerably greater than that found after the milder hydrolysis. In a few cases, particularly in the female urines, the amount of galactose also was increased on prolonged hydrolysis. When the proportion of fermentable to non-fermentable substance produced on hydrolysis of the copper-lime precipitate (Fraction C) was observed at frequent intervals during the course of hydrolysis (Fig. 1), it was

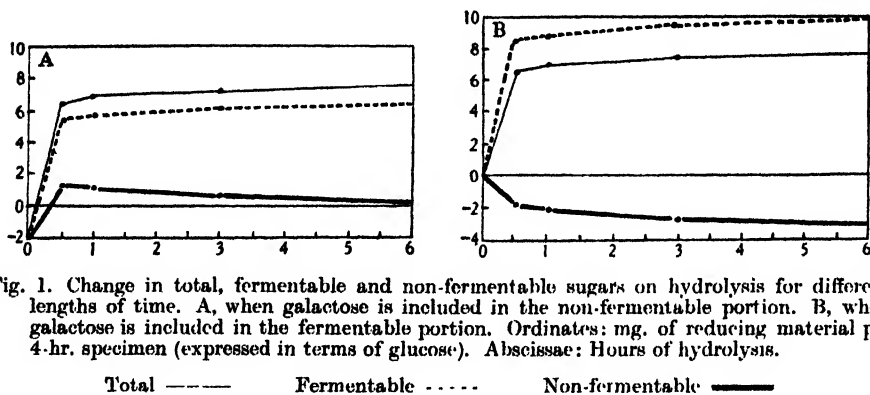


Fig. 1. Change in total, fermentable and non-fermentable sugars on hydrolysis for different lengths of time. A, when galactose is included in the non-fermentable portion. B, when galactose is included in the fermentable portion. Ordinates: mg. of reducing material per 4-hr. specimen (expressed in terms of glucose). Abscissae: Hours of hydrolysis.

found that there was a constant decrease of the non-fermentable portion of the filtrate. If the fermentable and non-fermentable fractions were determined by the use of baker's yeast this decrease might be masked during the first hours of the procedure by the production of galactose which would be included in the non-fermentable portion. Mild hydrolysis of the copper-lime filtrate (Fraction H) gave no appreciable increase in total reducing power and produced extremely small amounts of fermentable material. The prolonged hydrolysis (Fraction J) produced a slight increase in reducing value. The fermentable sugar produced was glucose.

The nature of the precursor of the sugar produced on hydrolysis is still unknown. That some portion of it is possibly lactose is shown in the next paper [Harding *et al.* 1936]. The remaining portion is also for the most part non-

¹ It has been suggested by Laug and Nash [1935] that the fermentable sugar found in $\text{HgSO}_4\text{-BaCO}_3$ filtrates might be formed by the hydrolytic action of the strong acid in the reagent on some precursor of the fermentable sugar. Even if this were true for dog's urine, it does not seem at all probable that the fermentable sugar found in human urine originates thus, in view of the findings of Harding and Selby [1933]. These workers found significant amounts of fermentable sugar in fasting human urine when they used the $\text{HgSO}_4\text{-BaCO}_3$ method to prepare their urine filtrates. This fermentable sugar could not however have been produced by any hydrolytic action on the part of the clearing reagent since the fermentations were carried out on the diluted urines before clearing. In the same paper one comparison was made between the fermentable sugar found when the fermentation was done before the urine was cleared and that found when the fermentation was done on cleared urine. No significant difference was noted when $\text{HgSO}_4\text{-BaCO}_3$ was the clearing reagent used.

nitrogenous since the copper-lime precipitate, which is nitrogen-free, contains by far the larger portion of the substances giving fermentable material on hydrolysis.

Recently Laug and Nash [1935] suggested that this precursor of fermentable sugar was non-reducing and non-fermentable and that on hydrolysis it produced first a non-fermentable reducing material and on further hydrolysis a fermentable reducing substance. The relationship between total, fermentable and non-fermentable reducing substances shown in 6-hour hydrolysis curves, the presence of an initial rise in the non-fermentable portion and the fact that the total non-fermentable did not in most cases drop below the level found before hydrolysis were the basis for the theory. Nash presumably determined the amount of fermentable sugar present in the hydrolysates by the use of baker's or brewer's yeast, which does not remove galactose. This sugar would therefore be included in the non-fermentable fraction. An entirely different explanation of the results becomes evident when galactose is included in the fermentable portion. In Fig. 1 are shown curves obtained by determining glucose (including small amounts of fructose and/or mannose), galactose and non-fermentable reducing substances. If galactose is included in the non-fermentable fraction curves similar to those of Laug and Nash are obtained. Otherwise there is in at least 50 % of the urines examined a steady decrease in the non-fermentable portion throughout the period of hydrolysis. It is probable therefore that a considerable proportion of the fermentable sugar produced on hydrolysis comes from the non-fermentable reducing material.

Everett *et al.* [1934] and Laug and Nash [1935] present indirect evidence favouring the view that most of the sugar in urine filtrates is non-nitrogenous. Since repeated copper-lime precipitation gave a nitrogen-free fraction containing most of the reducing material, we are able to support the conclusions of these workers with evidence which is somewhat more direct.

SUMMARY.

1. By application of a modification of the Salkowski copper-lime method for the precipitation of carbohydrates to filtrates of "composite fasting" urine treated with basic lead acetate and mercuric sulphate-barium carbonate, concentrated solutions of the carbohydrate material were prepared.

2. Differential fermentation of these solutions showed the presence of glucose and galactose both before and after hydrolysis. Very small amounts of fructose or mannose were found in some urines.

3. It is probable that the greater part, if not all, of the "fermentable" sugar produced on hydrolysis is derived from the non-fermentable reducing fraction of the urine. The postulation of a non-reducing precursor is unnecessary if galactose is included in the "fermentable" fraction.

4. By far the greater part of the "sugar" in urine is non-nitrogenous.

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LII. SOME PROPERTIES OF THE REDUCING MATERIAL IN CERTAIN FRACTIONS OF NORMAL URINES.

II. (a) THE EFFECT OF CERTAIN "TYPE MEALS" ON THE "HYDROLYSABLE SUGAR" IN URINE.

(b) SOME FURTHER EVIDENCE AS TO THE NATURE OF THE HYDROLYSABLE SUGAR IN URINE.

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IN the preceding paper Harding *et al.* [1936] presented evidence to show that fasting urine contained non-fermentable substances which on hydrolysis yielded glucose and small amounts of galactose and of fructose and/or mannose. Some evidence was presented which indicated that at least a portion of the hydrolysable sugar was reducing sugar but nothing more regarding the nature or source of the hydrolysable sugar was determined. West *et al.* [1932] showed that all types of food produced some increase in the hydrolysable carbohydrates excreted, the amounts of the hydrolysable sugar excreted being similar with all types of foods except those containing caramelised carbohydrates, these latter causing a very much greater increase in the excretion of fermentable, non-fermentable and hydrolysable sugars than any of the others. These authors believed that the hydrolysable sugar in urine was non-reducing as did Laug and Nash [1935]. Harding and Selby [1931] showed that the nature of the midday meal had a marked effect on blood sugar curves determined 4 hours after the ingestion of a "type meal" of carbohydrate, protein or fat. There was no effect on the fermentable sugar excreted in the urine for a 4-hour period following the meal. The effects of these "type" meals on the excretion of hydrolysable sugar in the urine were not studied. It was thought that an investigation of the effect of such meals on the amounts of non-fermentable hydrolysable sugars excreted in the urine and on the proportions of the various fermentable sugars released on acid hydrolysis might yield further information as to the source of the hydrolysable sugar.

A comparison of the amount of sugar produced by the hydrolysis of "Lloyd's" and " $\text{HgSO}_4\text{-BaCO}_3$ " [West *et al.*, 1929] filtrates was made in order to determine whether or not any large proportion of fermentable sugar was contained in compounds (possibly nitrogenous in nature) which were precipitable by mercury but not by Lloyd's reagent.

More direct evidence as to the nature of the hydrolysable sugar was sought by subjecting one portion of urine to the action of hypiodite before hydrolysis and comparing the fermentable sugars produced on hydrolysis of the portion so treated with those liberated from another portion which had not undergone the

hypoidite treatment. By this means it was possible to estimate what proportion of the fermentable material liberated from the hydrolysable material was so combined that it presented a free aldehyde group.

Analytical methods.

In order to get sufficient urine for analysis the urines of two individuals were combined in each of the series of tests made. The individuals ate a light breakfast of orange juice, cereal, bacon, toast and coffee. At noon the "type" meal was taken. The "fat", "starch" and "protein" meals were similar to those described by Harding and Selby [1931]. The "fruit" meal consisted of mixed fresh fruits—oranges, grapefruit and apples—to give about one-half the amount of total carbohydrate present in the "carbohydrate" meal. In addition, tests were made on urines collected when no noon meal was taken and in the morning, 12 hours after the last meal. The urine passed in the 4 hours following the taking of the "type" meal was collected. In the case of the 12-hour fasting specimen, the urine was collected between 7 and 11 a.m. When no noon meal was taken the urine was collected between noon and 4 p.m. The combined urines were made up to a volume of 500 ml. thus facilitating calculations by giving a constant volume to the 4-hour specimen. "Fermentable" sugar was removed by incubating 325 ml. of the urine for 10 min. at 38° with 10 g. wet weight of baker's yeast (Fleischmann's) prepared according to Somogyi's [1927] directions. After incubation the yeast was removed by centrifuging. 100 ml. of the fermented urine specimen were cleared with Lloyd's reagent as described by Harding and Selby [1931] and 200 ml. were cleared by the use of basic lead acetate¹ followed by $\text{HgSO}_4\text{-BaCO}_3$, using the same proportions of reagents to urine and the same methods of removing the excess reagents from solution as were specified in the preceding paper. In both instances the urines were diluted 1:2 in the process of clearing. Each filtrate was then treated in an identical way. The largest convenient aliquot was again diluted 1:2, sufficient 5.0 N H_2SO_4 being added in the course of dilution to give N concentration. The acidified filtrate was boiled for 4 hours under a reflux condenser. After cooling the solution was neutralised with CaCO_3 . The precipitated CaSO_4 was removed by suction filtration and the filtrate again

¹ The maximum amount of basic lead acetate that may be used without the removal of fermentable sugar is 1 ml. of 0.5 N solution to 8 ml. of urine. The time that the basic lead acetate is in contact with the urine is of importance. If the precipitate is filtered off immediately, a considerable loss of fermentable sugar occurs, nor can added sugars be completely recovered. If on the other hand the precipitate and filtrate are allowed to remain in contact (with occasional shaking) for 30 min. no loss of fermentable sugar occurs and added sugars may be recovered quantitatively. Some sugar is evidently occluded in the precipitate and later diffuses out into the filtrate. The use of this procedure before the $\text{HgSO}_4\text{-BaCO}_3$ decreases the total nitrogen of such urine filtrates by from 45 to 55%, lowers the total reducing value of the filtrate by from 15 to 20% and completely removes the substance which is responsible for the secondary removals by successive yeast treatments, as in the Van Slyke and Hawkins [1929] phenomenon which was observed in concentrated $\text{HgSO}_4\text{-BaCO}_3$ filtrates of urine by Harding *et al.* [1936]. The decrease of nitrogen in the lead-mercury filtrates as compared with the filtrates obtained by the use of the $\text{HgSO}_4\text{-BaCO}_3$ alone was due to a decrease in the urea content of the filtrate as neither filtrate contained any organic non-urea nitrogen. Since basic lead acetate itself has little effect on urea this reduction in urea content was probably due to the action of the lead acetate in removing non-urea-nitrogen and thus allowing more complete precipitation of the urea by the $\text{HgSO}_4\text{-BaCO}_3$ procedure. Similar reductions in the urea content of the filtrates may be obtained by a second treatment with $\text{HgSO}_4\text{-BaCO}_3$ but this decreases the amount of filtrate very markedly and has no effect on the substance which gives the successive removals on repeated yeast fermentation.

treated with $\text{HgSO}_4\text{-BaCO}_3$, 20 ml. of the HgSO_4 solution being used for every 100 ml. of filtrate, and a further dilution of 1 : 2 being effected, to remove the charred material produced during the hydrolysis.

The reaction of the filtrate was adjusted to p_H 6.5 with a drop of 50 % KOH and the filtrate concentrated by evaporation under reduced pressure at a temperature of 37° to about 25 ml. The concentrate was subjected to the modified Salkowski [1879] copper-lime precipitation procedure [Archibald, 1935] described in the preceding paper.

The regenerated copper-lime precipitate which represented a dilution of 1 : 1.5 of the original 500 ml. 4-hour specimen was analysed by the mycological method of Harding and Nicholson [1933] to determine the amounts of the various sugars that had been liberated by hydrolysis.

Another group of combined urines passed in the 4-hour period from noon to 4 p.m. by individuals who had taken their ordinary breakfast and lunch and a further group collected from 8 a.m. to noon following a breakfast consisting solely of a quart of skimmed milk were cleared by the basic lead acetate- $\text{HgSO}_4\text{-BaCO}_3$ method. The filtrate from each "combined" urine was then divided to give two 100 ml. portions. One portion was treated in the same manner as the other lead acetate-mercury filtrates. The other portion was neutralised with 40 % NaOH and 15 ml. of 0.05 *N* I solution (made up in as dilute KI as possible) was added. 0.1 *N* NaOH was added drop by drop, with stirring, at such a rate that not more than 0.5 ml. was added per min. The addition was continued until the solution was almost colourless (about 15 ml. were usually required). After 10 min. the solution was acidified with 3 ml. of 5 *N* H_2SO_4 and aerated vigorously for 2 hours to drive off as much of the free iodine as possible. The remaining iodine and any iodides or iodates were removed by the addition of 10 ml. of the HgSO_4 reagent. The volume was then made up to 200 ml. and the Hg removed with BaCO_3 followed by H_2S . The resulting solution was then hydrolysed and treated in the same manner as the other filtrates.

All determinations of reducing material were made with the modified Shaffer-Hartmann reagent of Harding and Downs [1933] using a 10-min. boiling period.

The effect of "type meals" on the hydrolysable sugar in urine.

Tables I and II give typical results obtained after hydrolysis of "Lloyd's" and "lead acetate-mercury" filtrates. All the meals produced marked increases in hydrolysable sugar over the fasting specimen but the true effects of the type meals are seen by comparison with the urines collected in the afternoon when a normal breakfast had been taken but no noon meal given. There was a noticeable increase in the excretion of substances giving rise to fermentable sugar on

Table I. *Fermentable sugar produced on hydrolysis.*

Sugar as mg. glucose per 4 hours.

	Total fermentation		Glucose		Fructose and/or mannose		Galactose	
	Lloyd's	Pb Ac. HgSO_4	Lloyd's	Pb Ac. HgSO_4	Lloyd's	Pb Ac. HgSO_4	Lloyd's	Pb Ac. HgSO_4
12-hr. fasting	7.3	4.0	5.5	2.6	0.3	0.0	1.5	1.4
No noon meal	20.8	15.1	15.1	9.6	0.0	0.0	5.7	5.5
Fat	25.0	24.8	18.0	18.2	2.0	1.6	5.0	5.0
Fruit	28.9	27.0	21.0	19.6	3.3	3.0	4.6	4.4
Starch	25.2	20.2	17.5	13.6	3.1	2.0	4.6	4.6
Protein	20.6	15.4	13.0	8.8	3.1	1.8	4.5	4.8

Table II. *The fermentable sugars produced by hydrolysis before and after hypiodite oxidation of "fermented" filtrates of urine passed after mixed meal.*

		Sugars as mg. glucose per 4 hours		
		Direct hydrolysis	Hydrolysis after hypiodite oxidation	Loss of sugar due to hypiodite oxidation
A	Fructose and/or mannose	4.8	3.8	1.0
	Glucose	21.6	14.4	7.2
	Galactose	5.1 (7.0)*	5.7 (7.5)*	—
B	Fructose and/or mannose	1.7	2.2	—
	Glucose	15.4	8.1	7.3
	Galactose	2.1 (2.9)*	1.7 (2.4)*	0.5

* mg. galactose per 4 hours as galactose.

hydrolysis (shown by the increase in fermentable sugar produced on hydrolysis) in all instances except after the protein meal. These substances will be referred to for convenience as "hydrolysable sugar". The increase in all cases was due principally to an increase in the glucose-containing portion but there was also a definite excretion of some fructose-containing substance which was not present in the 12-hour fasting or in "no noon meal" specimens. The amounts of fructose found did not vary markedly although there was slightly more after the "fruit" meal than after any of the others. The amount of galactose found was fairly constant, there being little change in the amount excreted after any of the meals as compared with the afternoon specimen collected when no noon meal was given. There was however a very considerable increase over the fasting value. The amount of galactose found evidently depended on the morning meal and was probably due to excretion of a portion of the lactose taken in the milk.

When fermentable sugars resulting from the hydrolysis of the Lloyd's and the lead acetate- $\text{HgSO}_4\text{-BaCO}_3$ filtrates are compared, it is seen that the use of the lead-mercury method of clearing resulted in a definite decrease in the amount of glucose produced on hydrolysis in the "12-hour fasting" and "no noon meal" specimens and following the "starch" and "protein" meals. The difference in the cases of the "fat" and "fruit" meals was very small or non-existent. Hydrolysis of the lead-mercury filtrates gave rise to a smaller amount of fructose than did hydrolysis of the Lloyd's filtrates. The difference was small but definite and was observed in the urines passed after every type of meal. The galactose figure did not vary with the method of clearing used. It is evident that urine contains some fermentable sugar in substances (possibly nitrogenous in nature or more probably dextrin-like and corresponding to a portion of the alcohol-precipitable carbohydrate of Lustig and Landau [1932]) which are precipitable by lead acetate and mercury but not by Lloyd's reagent.

The immediate effects of the various meals on the total amount of hydrolysable sugar excreted were not great but "fat" and "fruit" meals had marked effects on the excretion of the lead-mercury-precipitable substance. There was almost complete suppression of the excretion of this substance for the 4-hour period following the ingestion of these two types of meals. It was remarkable that the glucose produced on hydrolysis of the specimen collected after the fat meal showed such a definite increase over that of the "no meal" specimen. This could not be due to specific dynamic action since protein, which has a much greater specific dynamic action, failed to increase any of the hydrolysable sugars. This effect might however be connected with the very striking effect of the fat meal on the glucose tolerance which was noted by Harding and Selby [1931]. The fact

that fruit, in which the sugars occur mainly as monosaccharides or simple polysaccharides that are readily hydrolysed in the digestive tract, increased the excretion of "hydrolysis" glucose even more than did complex polysaccharides such as starch, was also of considerable interest. The ingestion of protein had no immediate effect on the excretion of hydrolysable carbohydrate material, the total and the amount of lead-mercury-precipitable substance found being the same for the 4-hour period following the protein meal as for the similar period when no noon meal was taken.

Further evidence as to the nature of the hydrolysable sugar in urine.

West *et al.* [1932] and Laug and Nash [1935] have stated that the fermentable reducing sugar produced on hydrolysis of lead-mercury filtrates of urine was derived entirely from non-reducing polysaccharides. Harding *et al.* [1936] suggested that a part at least of the "hydrolysable" material was reducing in nature and that the failures of West *et al.* and Laug and Nash to detect any reduction of the non-fermentable portion of the urine filtrates after hydrolysis were due to the fact that liberation of galactose, which is not fermented by baker's yeast, masked the reduction in the non-fermentable reducing substances.

The use of hypiodite oxidation combined with acid hydrolysis gave more direct evidence as to the nature of the hydrolysable substances. In Table II are recorded two sets of results obtained on urine specimens following a mixed meal. Comparison of the amounts of the various sugars produced by acid hydrolysis before and after hypiodite oxidation shows that glucose was the only sugar affected. The galactose figure remained constant. It is evident that about $\frac{1}{3}$ to $\frac{1}{2}$ of the glucose was combined with the aldehyde group free whereas all the galactose was present in the form of galactosides. It seems quite possible that the galactose was present in the form of lactose. Lactose however could not account for all the glucose which might be combined to form a reducing material because the amount of galactose produced on hydrolysis did not in one instance (Table II B) equal more than about $\frac{1}{3}$ of the glucose oxidised by the hypiodite. In some urines therefore a very considerable proportion of the glucose produced on hydrolysis from reducing non-fermentable carbohydrates came from some source other than lactose.

The ingestion of a large amount of skimmed milk did not result in any increase in the total hydrolysable sugar but the proportion of galactose produced on hydrolysis was increased considerably. In Table III are recorded the findings following two "milk breakfasts". The galactose was again unaffected by the

Table III. *The fermentable sugars produced by hydrolysis, before and after hypiodite oxidation, of "fermented" filtrates of urines passed after "milk" meals.*

		Sugar as mg. glucose per 4 hours		
		Direct hydrolysis	Hydrolysis after hypiodite oxidation	Loss of sugar due to hypiodite oxidation
A	Fructose and/or mannose	2.8	1.3	1.5
	Glucose	15.3	6.7	8.6
	Galactose	8.0 (11.1)*	8.0 (11.1)*	—
B	Fructose and/or mannose	3.0	3.5	—
	Glucose	17.6	8.8	8.8
	Galactose	8.5 (11.8)*	8.2 (11.4)*	—

* mg. galactose per 4 hours as galactose.

hypiodite oxidation, as much having been produced on hydrolysis of the hypiodite-treated filtrates as by hydrolysis of the untreated filtrates. The amount of galactose liberated exceeded the amount of glucose destroyed by a small but significant amount. Some galactose was therefore excreted combined through its reducing group in some sugar complex other than lactose.

These results confirm the conclusion of Harding *et al.* [1936] that, when fermentations are carried out with baker's yeast, the liberation of galactose may mask reductions in the non-fermentable reducing substances which occur on hydrolysis. If the figures in Table II A are examined it will be seen that the liberation of galactose could maintain the non-fermentable figure at the same level as before the urine filtrate was hydrolysed. On the assumption that all the glucose with its reducing group free was combined as lactose there would be 13.7 mg. of lactose present in the filtrate which would give a titration figure equivalent to 5.3 mg. of glucose. As the galactose liberated (7.2 mg.) would be equivalent to 5.2 mg. glucose there would be no change in the reducing value of that portion of the filtrate which was not fermentable by baker's yeast.¹ Following a milk meal, assuming again that all the glucose oxidised by the hypiodite was combined as lactose, there would be an actual rise in the non-fermentable reducing substances on hydrolysis of the filtrate if the galactose produced were not fermented.

SUMMARY.

1. All of the various "type" meals—"fat", "fruit", "starch" and "protein" (with the exception of the last-named)—caused increases in the amounts of the substances giving rise to fermentable sugar on hydrolysis excreted in the urine during 4 hours after the meals.
2. The increase was due almost entirely to glucose-producing substances.
3. A small amount of material which produces glucose on hydrolysis is precipitated by lead acetate- HgSO_4 - BaCO_3 treatment but not by Lloyd's reagent. This substance is present in urines collected after 12 hours' fasting as well as in non-fasting urines.
4. The excretion of the lead acetate- HgSO_4 - BaCO_3 -precipitable substance does not occur after "fat" or "fruit" meals.
5. An examination of the effect of hypiodite oxidation of the urine filtrates on the sugars produced by acid hydrolysis shows that about one-third of the hydrolysable material excreted is reducing in nature, the reducing group being in the glucose molecule.
6. All the galactose released on hydrolysis is combined through its reducing group, possibly in the form of lactose.
7. The reduction in the non-fermentable portion of the urine filtrates which would be expected following acid hydrolysis and yeast fermentation may be masked by the galactose liberated if the fermentation organism does not remove galactose.

¹ These figures refer to reductions obtained with the Harding-Downs reagent using a 10-min. boiling period (see Table I, Harding and Downs [1933]). Using the Shaffer-Somogyi [1933] reagent 50 containing 1 g. of KI per litre with a 15-min. boiling period, as employed by West *et al.* and by Laug and Nash, the results would be very similar, since lactose, under these conditions, gives only 57% of the reduction given by galactose.

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LIII. SULPHURETTED HYDROGEN AS A FACTOR IN THE DETERMINATION OF FREE AND BOUND AMYLASE IN UNGERMINATED CEREALS.

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THE question of the valuation of cereals in regard to total amylase content, even in the period before their germination, is very important both from the theoretical and the practical points of view. It is well known that apart from free amylase, which can be extracted by the use of water from ungerminated cereals, there are also large quantities of bound amylase which are inactive and which cannot be extracted, but which are freed during the process of germination, excluding of course from consideration the possibility of the formation of new quantities of amylase during this process. In order to determine the content of bound amylase, papain is usually added [Ford and Guthrie, 1908]. Our researches have shown that the action of papain can be partially replaced by that of eluants [Chrząszcz and Janicki, 1933], especially in the case of malt. It has further been shown that apart from papain, there are other proteolytic enzymes which can be used, trypsin being a special case in point [Chrząszcz and Janicki, 1934, 1, 2]. The present authors have also shown [1935] that chymosin (rennin) can likewise be used for the determination of bound amylase content in ungerminated cereals as this, especially with some cereals, has a greater influence upon increases in the active amylase than papain. In addition, it has been demonstrated in the papers referred to above that the decomposition of the proteins of the various cereals depends on the type of enzyme used, this having been shown particularly in the case of the decomposition of the protein of oats by rennin [1935]. Following this line of approach we were able to extend the observations of Blagovestshensky [1934] on the specific action of the proteolytic enzymes. If it be accepted that amylase is really bound by the protein of the ungerminated cereal, and that the protein of a given type of cereal is decomposed most strongly by the proteolytic enzyme of the same origin, then it might be expected that by using these enzymes the greatest quantity of bound amylase would be extracted. The researches of Mendel and Blood [1910], Willstätter and Grassmann [1924; 1925], and Grassmann and Dyckerhoff [1928] have demonstrated that the plant proteolytic enzymes become active under the influence of sulphuretted hydrogen. The researches of the present authors upon the influence of H_2S on the quantity of amylase in ungerminated cereals have shown that this gas must have a specific action on amylase [1935-36]. On this basis it proved possible to elaborate a method of determining the amylase content by the use of H_2S .

EXPERIMENTAL.

In the experiments, four samples of barley (Nos. 1-4), two of wheat and one of rye were taken, Merck's soluble starch (Erg. B 5) and papain (1:100), were used.

Preparation of aqueous extracts. 25 g. of ground cereal + 150 ml. of H_2O + 4 ml. of toluene were left at room temperature (c. 18°) for 15 hours and shaken from time to time, or mechanically shaken at 60 r.p.m.

Preparation of extracts with addition of papain. The process followed was the same as above but with the addition of 3 g. of papain.

The H_2S method. 25 g. of the ground cereal + 150 ml. of H_2O + 4 ml. toluene were placed in a flask. The air present in this flask was driven out by sulphuretted hydrogen produced by a Kipp's apparatus, after which the extract was saturated with H_2S under pressure for 20 min., during which period it was well mixed about every 5 min. and then left under pressure for 15 hours and shaken as above.

The extracts were then filtered and their amylolytic power ascertained by determinations of the saccharifying, dextrinising and liquefying powers in accordance with the methods described in previous papers [Chrzyszcz and Janicki, 1935]. In view of the fact that the dextrinising and liquefying powers under the influence of papain and H_2S underwent very slight fluctuations, their values have not been given in the tables. The saccharifying power was determined in extracts diluted in the proportion of 10:100, and expressed in ml. $N/20$ iodine, corresponding to the amount of sugar produced under the given conditions by the amount of extract equivalent to 1 g. of the cereal. Blank experiments were made when determining the saccharifying power, taking into consideration the reducing power of the extracts themselves and of the starch solution. Using this method of applying H_2S it is necessary to carry out the determination of the saccharifying power immediately after filtration and dilution, as otherwise the H_2S will escape or be oxidised by the air and so a weaker saccharifying power will be recorded.

RESULTS.

Barley. In order to compare the influence of papain and of H_2S on the quantity of active amylase obtained from ungerminated barley, extracts were prepared from four samples. The extracts of barley Nos. 1-3 were prepared by shaking from time to time, whilst sample No. 4 was in addition mechanically shaken.

Table I. *Saccharifying power of barley.*

Extract	Extracts shaken from time to time						Extracts shaken mechanically			
	Sample No. 1		Sample No. 2		Sample No. 3		Sample No. 4			
	$N/20$ iodine ml.	In- crease %	$N/20$ iodine ml.	In- crease %	$N/20$ iodine ml.	In- crease %	$N/20$ iodine ml.	In- crease %	$N/20$ iodine ml.	In- crease %
Aqueous	296.0	—	405	—	278	—	223.6	—	132.6	—
H_2S added	884.0	198	863.2	113.1	559	101	681.2	205	374.4	182
Papain added	740.0	150	754	86	465	67	556.4	149	312.0	135

From the above table it can be seen that the extracts prepared with the addition of H_2S show a considerably greater increase in the active amylase than those of samples by the papain method. Further, it can be seen that the extracts prepared by shaking occasionally have a greater saccharifying power than those shaken mechanically.

Wheat and rye. The results of the action of H_2S in comparison with the influence of papain on the activation of bound amylase in the cases of wheat and rye are given in Table II.

Table II. *Saccharifying power of wheat and rye.*

Extract	Extracts shaken from time to time		Extracts shaken mechanically		Extracts shaken from time to time			
	Sample wheat No. 1		Sample wheat No. 1		Sample wheat No. 2		Sample rye	
	N/20 iodine ml.	In- crease %	N/20 iodine ml.	In- crease %	N/20 iodine ml.	In- crease %	N/20 iodine ml.	In- crease %
Aqueous	390.0	—	278.2	—	317.2	—	413.2	—
H ₂ S added	681.2	74.6	509.7	83.2	494.0	55.8	530.4	28.3
Papain added	645	65.4	577.2	107.1	450.0	41.8	512.2	24.0

It will be noted that both these types of cereal give similar results, *viz.* a greater increase in the amount of active amylase under the influence of H₂S than under that of papain. It also transpires that the extracts of these cereals possess greater amylolytic power when they are not shaken mechanically during their preparation. It therefore appears best to shake the extracts only from time to time and not continuously.

SUMMARY.

1. A method is described for the determination of bound amylase in ungerminated cereals by the use of H₂S.
2. The increase in the amount of active amylase, *i.e.* the extraction of the bound amylase, is greater in presence of H₂S than when the papain method is used.
3. The extracts should only be shaken from time to time during preparation.

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LIV. THE DISTRIBUTION OF LEAD IN HUMAN BONES.

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(Received January 7th, 1936.)

It has been shown [Tompsett and Anderson, 1935] that lead occurs in appreciable amounts in the tissues of persons with no history of exceptional exposure to lead. The highest concentrations of lead were found in bones (rib and vertebrae). The concentration of lead in the rib varied from 5.2 to 12.9 mg. Pb per kg. (wet tissue) and in the vertebrae from 4.2 to 14.7 mg. Pb per kg. (wet tissue). Lynch *et al.* [1934], in an examination of human tibiae and femora, found 14–146 mg. Pb per kg. (wet tissue). It was decided therefore to compare the concentrations of lead in the rib, the vertebrae, the shaft of the femur and the shaft of the tibia. These bones were obtained from cases of widely different ages having no history of special exposure to lead.

EXPERIMENTAL.

A weighed portion of the bone (tibia and femur 1–2 g.; rib and vertebrae 10–20 g.) was dried and incinerated in a silica basin containing 100 ml. of lead-free sodium phosphate. Lead was then determined as described previously [Tompsett and Anderson, 1935].

Table I.

The results are expressed in mg. Pb per kg. fresh bone.

	Age	Occupation	Diagnosis	Rib	Verte- brae	Femur	Tibia
			Males.				
1	25	Quarryman	Nephritis	5.9	4.6	33.9	27.8
2	28	Newsagent	Aspirin poisoning	9.8	6.4	25.6	27.0
3	29	Clerk	Ulcers of small in- testine	4.2	4.6	21.1	20.6
4	35	Boiler fireman	Carc. of stomach	13.3	7.3	48.2	28.6
5	50	Hairdresser	Carc. of stomach	7.9	4.6	48.1	42.1
6	58	Labourer	Nephritis	8.9	8.6	29.0	26.5
7	59	Engineer	Uraemia	12.8	11.6	70.0	62.7
8	66	Iron moulder	Carc. of pelvic colon	8.6	6.5	42.3	48.6
9	67	Retired	Cerebral haemorrhage	9.1	8.2	55.9	43.7
10	67	Retired	Fractured skull	11.1	12.4	108.3	96.5
11	68	Blacksmith	Carc. of bronchus	11.3	7.6	74.1	43.4
12	74	Restaurant proprietor	Coronary thrombosis	13.4	12.6	82.2	81.6
			Females.				
13	12	—	Malignant thymoma	4.0	3.4	19.1	17.9
14	19	Shop assistant	Subacute bacterial endocarditis	16.5	10.6	74.8	60.0
15	22	Clerk	Pernicious anaemia	7.2	6.2	18.2	15.3
16	30	Housewife	Burns	9.5	8.4	34.1	29.8
17	39	Housewife	Subacute bacterial endocarditis	9.8	10.6	44.5	46.8
18	49	Housewife	Toxic goitre	8.7	9.0	33.3	27.8
19	61	Housewife	Abscesses of the kidneys	17.5	16.5	61.7	61.5

The pink colours developed with diphenylthiocarbazone are quite stable unless exposed to bright sunlight when rapid fading will take place.

The results are shown in Table I.

From the results it will be seen that femora and tibiae contain much higher concentrations of lead than ribs and vertebrae. The concentrations of lead in tibiae and femora appear to be of the same order and agree with those reported by Lynch *et al.* [1934]. It seems that in cases of suspected lead poisoning, different types of bone should be examined.

SUMMARY.

1. Femora and tibiae contain much higher concentrations of lead than ribs and vertebrae.

2. In a series of 19 cases, the concentrations of lead were, rib 4.0–17.5, vertebrae 3.4–16.5, femur 18.2–108.3, tibia 15.3–96.5 mg. Pb per kg. fresh bone.

In conclusion I wish to thank Dr A. B. Anderson for his helpful criticism and advice.

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LV. STUDIES ON BILE PIGMENTS.

I. A STUDY OF EHRLICH'S TEST FOR UROBILINOGEN AND SCHLESINGER'S REACTION FOR UROBILIN.

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THE study of bile pigment derivatives is important because these substances represent the end-products of haemoglobin metabolism. The destruction of haemoglobin both normal and pathological can thus be measured by quantitative determination of the urobilin and urobilinogen in urine and faeces. On the other hand, when pathological destruction of haemoglobin can be excluded, urobilin- or urobilinogen-uria indicates failure of the liver to take up these substances from the portal circulation. Thus the test is valuable in the study of both blood and liver diseases. In the present work the Ehrlich-Neubauer colour reaction for urobilinogen with *p*-dimethylaminobenzaldehyde in HCl and the Jaffé-Schlesinger fluorescence reaction with zinc-acetate-alcohol for urobilin have been studied as qualitative urine tests.

Neubauer [1903] identified urobilinogen as the reacting substance in Ehrlich's aldehyde test but described also positive reactions with other pyrrole derivatives such as reduced haematin and haemopyrrole. Thomas [1907] summarised the chemical properties of urobilin and urobilinogen and mentions indole and tryptophan as interfering substances. Fischer and Meyer-Betz [1911; 1912] first isolated urobilinogen from a pathological urine and assumed this substance to be identical with mesobilirubinogen prepared from bilirubin. Watson [1932; 1933] and Lemberg [1934] however stated that urobilinogen is not identical with mesobilirubinogen which differs from the former in its characteristic violet iron reaction. The quantitative estimation has been greatly improved by Charnass [1909] who first applied Ehrlich's test quantitatively instead of using the less stable urobilin reactions. Terwen [1924] devised a quantitative method introducing ferrous salts for the reduction of urobilin.

EXPERIMENTAL.

Ehrlich's test with p-dimethylaminobenzaldehyde in HCl (aldehyde test).

For the application of the aldehyde test to urine it is essential to observe the following standardised conditions. Fasting urine should be used in order to avoid errors due to digestive urobilinogenuria and dilution of the urine. Even so a heavy meal as long as 2 days before can cause a marked increase of urobilinogen so that in doubtful cases the test should be repeated. The urine must be fresh or can be kept in the cold for a day in a dark bottle filled up to the cork. The reagent is prepared by dissolving 1 g. of pure *p*-dimethylaminobenzaldehyde in 100 ml. of conc. HCl and kept in a dropping bottle delivering 20 drops per ml. Neither dilution of the HCl nor the prescription of Niemann [1925] for avoiding the faint yellow tinge of the reagent is of advantage in testing the urine.

The final concentration of HCl in the urine is of the greatest importance for a correct test, since excess destroys the urobilinogen and the urobilinogen-aldehyde product and hydrolyses protein and indole derivatives transforming them into more reactive compounds. Since the reagent is made up in conc. HCl the final concentration of the latter will depend on the quantity of reagent added, since a single drop of the reagent provides a great excess of aldehyde for complete reaction; the effect of varying final concentrations of reagent may therefore be ascribed to variation in HCl concentration. In Table I is shown the effect of varying quantities of reagent on the degree of coloration as measured in an Autenrieth colorimeter with Terwen's standard solution. For the comparison of the urine test a compensatory trough filled with suitably diluted urine is used as a light filter. In column 1 the quantities of reagent added to 2 ml. of urine or faeces extract are shown, in column 2 the final concentrations of HCl in % and in columns 3-5 readings of the scale multiplied by the dilution rate.

The aqueous faecal extract and the urine with increased urobilinogen both show the highest figure on addition of 0.15 ml. of aldehyde reagent corresponding to a concentration of 2.5 % or 0.69 *N* HCl. Normal urine, however, shows no peak, but a steady increase up to a concentration of 7.2 % HCl which can be explained only by the hydrolysis of interfering substances. The latter play a minor rôle in both the other solutions with higher urobilinogen contents. The importance of interfering substances especially in normal urine may be demonstrated by carrying out the same procedure in ether extracts of the urines and faecal extracts; the readings obtained in this way were 42 for the faecal extract, 32 for the urine with increased urobilinogen and 17 for normal urine. Since urobilinogen is completely extracted by ether the difference in the results obtained can only be explained by the presence of interfering substances which are not extracted by ether.

Table I. *Influence of different concentrations of HCl on the aldehyde test.*

ml. of reagent	Final concentration of HCl %	Diluted aqueous extract of normal faeces	Diluted urine with increased urobilinogen	Normal urine
0.025	0.4	34	41	22
0.05	0.9	44	65	30
0.075	1.3	47	72	31
0.1	1.7	48	74	36
0.125	2.1	50	68	37
0.15	2.5	57	76	42
0.2	3.3	55	68	45
0.25	4.0	52	55	47
0.3	4.7	46	53	51
0.35	5.4	—	—	52
0.4	6.0	—	—	59
0.45	6.6	—	—	55
0.5	7.2	—	—	59

The effects of temperature and time factors on the aldehyde test are shown in Table II. The determination is the same as in Table I, 0.15 ml. of reagent being added to 2 ml. of aqueous faecal extract. The temperature was measured before and after each series of readings and the mean temperature given in the Table. The course of the aldehyde reaction is more rapid at higher temperatures but at the same time the destructive and hydrolytic effects of the acid increase at an even higher rate. It is therefore quite erroneous to boil the urine as is done sometimes, when a reaction fails to occur in the cold. Optimum conditions of

temperature and time appear to be 20° and 5 min. after mixing. Freshly passed urine is better cooled first to room temperature.

From the above results the following procedure is recommended for the aldehyde test:

0.15 ml. (3 drops) of 1% *p*-dimethylaminobenzaldehyde in conc. HCl is added to 2 ml. of fresh or suitably preserved urine at about 20° in a test-tube of 10 mm. diameter. The reaction is normal if after 5 min. an orange tinge is observed, increased if the colour is red, and negative if the colour is yellow.

Table II. *The aldehyde test at different times and temperatures.*

Times in minutes after addition of the reagent	11°	18°	29°	54°
0.5	13	22	24	34
1	17	31	34	49
1.5	21	34	37	40
2	27	38	40	36
2.5	30	38	40	34
3	31	39	42	30
3.5	34	39	41	28
4	36	40	—	30
4.5	37	41	—	27
5	36	41	—	27

The value of the aldehyde test in the urine is greatly restricted by its non-specificity in the presence of interfering substances (see Table III). The orange-coloured reaction of normal urine is caused by mixed red and yellow reactions. The two different phases of this reaction can be observed more distinctly by employing a ring test and adding the aldehyde reagent underneath the urine by means of a capillary tube. A yellow ring appears which after a time becomes

Table III. *The most important substances interfering with the aldehyde test in the urine.*

Substances	Colour of reaction
Indole	Red
Indican	Yellow
Skatole	Blue
Protein and protein derivatives	Red-orange to violet
Tryptophan	Yellowish to reddish
Pyrrole and pyrrole derivatives	Red
Urea	Yellow
Salvarsan	Yellow
Extr. Rhei	Brown
Phenazone	Salmon-coloured
Aldehydes and hexamethylenetetramine	Inhibiting the urobilinogen aldehyde reaction

reddish at the lower surface. The yellow reaction is mainly due to urea and indican, the red to urobilinogen and indole. In cases of complete obstruction of the bile duct only a yellow reaction appears, but by using the ring test a faint reddish tint can be found which can be attributed to indole alone. A positive aldehyde test can be masked by pigments which do not themselves react such as bilirubin or drugs such as Extr. Sennae. On the other hand a reaction can be inhibited by the presence of aldehyde compounds such as formaldehyde, acetaldehyde and hexamethylenetetramine which splits off formaldehyde in the presence of acids. Such inhibiting substances are present also to a small extent in normal urine, as it was found that the reaction of aqueous faecal extract is decreased by addition of normal urine and very markedly by that of some

pathological urines. In the presence of albumin the reaction is delayed for hours and gives finally a strong brownish or violet colour. These facts demonstrate that the aldehyde test is not sufficient for the identification of urobilinogen in the urine.

The Jaffé-Schlesinger test for urobilin.

This test is based on the green fluorescence produced by zinc acetate in alcohol with urobilin. The same precautions regarding the general conditions of the urine as were given previously for the aldehyde reaction should be observed. The original direction by Schlesinger [1903] to use as reagent a suspension of 10 % zinc acetate in absolute alcohol appears most suitable. Neither the modification given by Adler [1922] nor that given by Elzso [1934] gives better results. In order to perceive smaller degrees of fluorescence the urine-alcohol filtrate must be viewed in a sufficiently deep fluid column against black velvet with a strong side light and compared with a control tube of approximately the same shade, wherein the fluorescence is eliminated by acidification. The test is carried out in the following way:

10 ml. of fresh or suitably preserved urine are mixed with the same vol. of a well-shaken suspension of 10 % zinc acetate in absolute alcohol and filtered. Two equal portions of the filtrate are poured into test tubes of 10 mm. diameter to a height of 10 cm. and to one of them are added a few drops of HCl. Both tubes are observed on black velvet with a strong side light the upper limit of the luminous cone being about 1 cm. below the surface. A slight bluish Tyndall phenomenon is sometimes observed, but only a greenish tinge indicates a positive reaction. Then a drop of $N/10$ iodine is added to each tube, transforming the urobilinogen that may be present into urobilin.

By means of this technique a faint fluorescence can be detected always in normal fasting urine even without addition of iodine. Probably this small amount of urobilin is produced from urobilinogen during the test. As to specificity the only similar reaction known is a red fluorescence of pure bilirubin with zinc acetate-alcohol and iodine as described by Auché [1908]. In applying the Jaffé-Schlesinger test to urine however interference by bilirubin does not occur, nor does a higher content of other pigments prevent the perception of even a faint fluorescence. The only significant interfering substance is albumin which adsorbs small amounts of pigments. Thus it is evident that for detection of both urobilin and urobilinogen the fluorescence test is more reliable than the aldehyde test. If both tests are used in conjunction a positive fluorescence test will prove that a positive aldehyde reaction is due actually to urobilinogen.

SUMMARY.

1. The conditions of Ehrlich's aldehyde test in fasting urine regarding concentration of the reagent, temperature and time have been investigated and a suitable technique has been devised.
2. The non-specificity of the aldehyde reaction has been emphasised and the most important interfering substances have been mentioned.
3. The orange-coloured reaction of normal urine has been explained as combined yellow and red reactions, the former being due mainly to urea and indican, the latter to urobilinogen and indole.
4. A suitable technique for the Jaffé-Schlesinger test has been devised so that traces of urobilin and urobilinogen can be detected in normal urine.
5. The fluorescence test has been found to be specific and therefore more reliable than the aldehyde reaction.

I wish to record my best thanks to Dr Arthur Davies, Director of the Devonport Pathological Laboratories of Seamen's Hospital, Greenwich, for his help and interest in my work, to the Seamen's Hospital Society for their assistance and hospitality to me and my assistant Miss Larissa Frenkel, whose co-operation has been of value and to the Academic Assistance Council for a personal grant.

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LVI. THE DETERMINATION OF SULPHATE. A STUDY OF THE CONDITIONS NECESSARY FOR THE PRECIPITATION OF BENZIDINE SULPHATE, WITH SPECIAL REFERENCE TO THE ESTIMATION OF SULPHATES IN URINE.

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THE chief difficulties in the benzidine method for determination of sulphate arise in obtaining the conditions requisite for maximum precipitation of benzidine sulphate. Most investigators have drawn attention to the importance of adjusting the acidity of the solution, while some of them have studied the interference known to be caused by certain ions: chlorides, for example, may increase the solubility of benzidine sulphate [Fiske, 1921; Kahn and Lieboff, 1928].

Undoubtedly the phosphate ion has been the source of most error. The earlier workers, Raschig [1903; 1906], Friedheim and Nydegger [1907], Järvinen [1912], Gauvin and Skarzynski [1913], Rosenheim and Drummond [1914], Raiziss and Dubin [1914] and Drummond [1915], considered that approximate adjustment of the reaction of the solution to p_{H} 2 by addition of hydrochloric acid would eliminate precipitation of benzidine phosphate. However Fiske [1921], Yoshimatsu [1926], Hubbard [1927; 1930], Pohorecka-Leslesz [1927], Chatron [1931, 1, 2], Power and Wakefield [1931], Letonoff and Reinhold [1934], and Öllgaard [1934] found it advisable to remove phosphate. Quite recently however Kahn and Lieboff [1928] and Friedrich and Bauer [1934] reported that they found it unnecessary to remove phosphate. There is also some difference of opinion as to the best method of washing a benzidine sulphate precipitate.

The present work consists of an attempt to standardise the method of analysis, the chief points studied being:

- (a) the influence of reaction on the precipitation of benzidine sulphate and phosphate;
- (b) the influence of chlorides in affecting the solubility of benzidine sulphate;
- (c) the influence of the concentration of benzidine hydrochloride;
- (d) the influence of different methods of washing the precipitate.

EXPERIMENTAL.

The effect of hydrogen ion concentration on the precipitation of benzidine sulphate.

Benzidine hydrochloride was used to precipitate solutions of pure sodium sulphate (B.D.H. Analytical Reagent). Various hydrogen ion concentrations in the reaction mixture were attained by prior addition of either acetic acid-sodium acetate buffers or hydrochloric acid. The benzidine sulphate precipitated

after 10 min. standing was titrated with 0.02 *N* CO₂-free NaOH, using phenolphthalein as indicator. All pertinent quantitative analytical details are given in the tables of results. Filtrates from all determinations were collected and preserved in stoppered Erlenmeyer pyrex flasks. The p_H of each filtrate was determined using a Hildebrand hydrogen electrode, a saturated potassium chloride bridge and a Cambridge (Gallenkamp) potentiometer. Such apparatus makes possible the determination of p_H to within 0.1 unit.

Separation and washing of benzidine sulphate is a matter of some difficulty. Filtration by suction, unless very carefully controlled, may easily lead to a "caked" precipitate which dissolves only with difficulty in boiling alkali, thus causing great loss of time and uncertain end-points.

Filtering with suction on Jena 10 G 4 sintered glass filters is rapid and convenient, but requires so much attention as to become very tedious when many estimations are being done. Filtering on paper has the disadvantage that the paper has to be transferred to the titration flask with the precipitate; the disintegrated filter-paper absorbs the indicator and makes it difficult to judge the end-point.

Special filter-tubes were therefore devised made of 1 in. hard glass tubing drawn out at one end, to which 1 in. of $\frac{3}{8}$ in. glass tubing was fused, making a total length of 6 in.

Each tube was prepared for filtration as follows. A loosely fitting ball of glass-wool was placed in the tube and pushed into the narrow part by means of a brass wire 8 or 9 in. long and slightly narrower than the bore of the narrow tube. Strands of glass-wool projecting into the wider part of the tube were broken off by pressing the brass wire against the shoulder of the joint and rotating it. These strands were then shaken out of the tube.

Too little glass-wool will not grip the sides of the narrow tube, whilst too much will form a hard comminuted plug through which water will flow too slowly.

The plug was pushed along the narrow tube until it was about $\frac{1}{8}$ in. from the shoulder.

A properly made plug allowed water to flow through under gravity as a rapid succession of drops.

A suspension of ashless filter-paper in distilled water was poured into the filter-tube so as to fill $\frac{3}{4}$ –1 in. above the plug. The tube was then filled with distilled water which was sucked through at the pump. Once all the water had passed through, a strong suction was applied in order to pack the paper-pulp tightly on top of the glass-wool. The rate of flow of the water was again tested. If the rate was about 2 drops per second the tube was ready for use.

The solution to be filtered was poured into the tube, the stream being directed against the side by means of a stirring rod. The tube, filled to within $\frac{1}{2}$ in. of the top, holds some 40 ml. For convenience in stirring the precipitate during subsequent washing, a stirring rod may be made with a hook about 1 $\frac{1}{2}$ in. from its upper end so that it may hang within the tube, without resting upon the filter-plug.

In the present determinations the filtrates were collected in 100 ml. Erlenmeyer flasks and tightly stoppered pending estimation of p_H .

As previous investigators have found, the precipitate could not be washed with water without considerable loss. Washing with 95 % acetone [Fiske, 1921] presented no advantages over washing, as recommended by Rosenheim and Drummond [1914], with a saturated solution of benzidine sulphate in water, 30–40 ml. of which, applied in two portions, were adequate.

After washing, the precipitate was transferred to a wide-mouthed flask by inverting the filter-tube so that its wide end was well inside the flask and dislodging the glass-wool plug by means of a rod. Both plug and precipitate were washed into the flask by means of a brisk jet of water from a wash-bottle. The tube was again inverted, scrubbed thoroughly with a rubber-tipped stirring rod and washed into the flask. Scrubbing and washing were repeated once.

The contents of the flask were then made up to 100 ml. with distilled water, phenolphthalein was added and the liquid titrated against 0.02 *N* NaOH as already indicated.

Blanks were subtracted from all determinations.

Results.

As will be seen from Tables I and II and Fig. 1 plotted from the data therein, variations in p_H between 1 and 5 exercise an effect upon the precipitation of benzidine sulphate.

Table I. *Effect of p_H on precipitation of benzidine sulphate.*

(Concentration of SO_4 before precipitation is the same as that of a normal human 24-hour urine specimen. Excess of benzidine is 100% in each case.)

No.	Vol. Na_2SO_4 sol. ml.	Vol. of buffer		Vol. of water ml.	Vol. of benz. reagent 8 g./l. ml.	Sulphate		° error	p_H of filtrate
		M Na acetate ml.	M acetic acid ml.			mg. S present	mg. S found		
1	20	5.6	2.4	12	20	13.35	12.99	- 3.1	4.7
2	20	5.6	2.4	12	20	13.35	13.02		4.7
3	20	3.2	4.8	12	20	13.35	13.04		4.1
4	20	3.2	4.8	12	20	13.35	13.20	2.5	4.2
5	20	1.6	6.4	12	20	13.35	13.46	- 0.5	3.6
6	20	1.6	6.4	12	20	13.35	13.28		3.6
7	20	0.2	7.8	12	20	13.35	13.36	0	2.15
8	20	0.2	7.8	12	20	13.35	13.33		2.15
9	20	—	—	20	20	13.35	13.28	0	2.0
10	20	—	—	20	20	13.35	13.36		2.0
N HCl added, ml.									
11	20	4	—	16	20	13.35	13.22	- 0.5	1.25
12	20	4	—	16	20	13.35	13.38		1.25
13	20	8	—	12	20	13.35	13.06	- 2.0	0.94
14	20	8	—	12	20	13.35	13.12		0.96

In the experiments recorded in Table I the concentration of sulphate in the solution used was the same as that in a 24-hour specimen of normal human urine. It will be seen that the addition of 0.5 ml. of 2.5 *N* HCl to 5 ml. of the solution analysed (the minimum amount required to hydrolyse the ethereal sulphates of urine [Drummond, 1915]) is sufficient to reduce the p_H of the reaction mixture to 1 and to cause an error of - 2% (approx.).

In more dilute solutions the effect of p_H on percentage recovery of sulphate is more marked (Table II).

This is further shown in the experiments in Table III. Here the p_H and the amount of benzidine hydrochloride used are constant, and the percentage of $SO_4^{=}$ precipitated diminishes as the concentration of the sulphate becomes less.

Table II. *Effect of p_H on precipitation of benzidine sulphate from dilute sulphate solution.*(Concentration of SO_4^{2-} before precipitation was one-quarter of that of a 24-hour specimen of normal human urine. Excess benzidine 108% in each experiment.)

No.	Vol. of Na_2SO_4 sol. ml.	Vol. of buffer		Vol. of water ml.	Vol. of benz. reagent 8 g./l. ml.	Sulphate		% error	p_H of filtrate
		<i>M</i> Na acetate ml.	<i>M</i> acetic acid ml.			mg. S present	mg. S found		
1	20	5.6	2.4	27	5	3.358	1.78	- 47.0	4.8
2	20	5.6	2.4	27	5	3.358	1.82	- 46.0	4.8
3	20	3.2	4.8	27	5	3.358	2.53	- 24.7	4.3
4	20	3.2	4.8	27	5	3.358	2.54	- 24.4	4.2
5	20	1.6	6.4	27	5	3.358	3.08	- 8.4	3.8
6	20	1.6	6.4	27	5	3.358	3.07	- 8.5	3.8
7	20	0.8	7.2	27	5	3.358	3.24	- 3.5	3.3
8	20	0.8	7.2	27	5	3.358	3.27	- 3.0	3.3
9	20	0.2	7.8	27	5	3.358	3.30	- 2.1	2.55
10	20	0.2	7.8	27	5	3.358	3.29	- 2.1	2.55
N HCl added, ml.									
11	20	None		35	5	3.358	3.28	- 2.5	2.3
12	20	None		35	5	3.358	3.28	- 2.5	2.3
13	20	2		33	5	3.358	3.02	- 10.2	1.34
14	20	2		33	5	3.358	3.00	- 10.8	1.34
15	20	4		31	5	3.358	2.62	- 22.0	1.1
16	20	4		31	5	3.358	2.65	- 21.3	1.1
17	20	8		27	5	3.358	1.92	- 43.3	0.85
18	20	8		27	5	3.358	1.94	- 42.8	0.85
19	20	8		27	5	3.358	1.92	- 43.3	0.84
20	20	35		0	5	3.358	0	- 100	0.21
21	20	35		0	5	3.358	0	- 100	0.21

Table III. *Effect of concentration of sulphate on benzidine sulphate precipitation.*(Concentration of benzidine, before precipitation, is kept constant, as also is p_H .)

No.	Vol. of Na_2SO_4 sol. analysed ml.	Vol. of benz. reagent ml.	Sulphate				p_H of filtrate
			mg. S/100 ml. present	mg. S/100 ml. found	% error	mg. S/l. remaining in sol.	
1	40	10	166.9	165.6	- 0.8	1.4	2.14
2	40	10	166.9	164.5	- 1.5	2.6	2.13
3	40	10	84.0	84.6	+ 0.7	—	2.15
4	40	10	84.0	83.3	- 0.8	0.7	2.13
5	40	10	41.8	39.9	- 4.5	1.9	2.15
6	40	10	41.8	40.1	- 3.9	1.6	2.20
7	40	10	41.8	39.8	- 4.8	2.0	2.18
8	40	10	21.0	18.3	- 12.9	2.7	2.05
9	40	10	21.0	19.0	- 9.6	2.0	2.05
10	140	35	8.4	6.95	- 16.8	1.4	2.26
11	140	35	8.4	7.13	- 14.6	1.3	2.27

Effect of excess of benzidine hydrochloride.

It should be emphasised here that the final concentration of benzidine hydrochloride remaining in excess is important independently of p_H in affecting the completeness of precipitation. Thus the shape of each curve in Fig. 1 is determined by p_H , but the position of curve *B* in relation to curve *A* is due to the fact that the excess of benzidine in the case of curve *A* is four times as great as in the case of curve *B*.

For the purposes of sulphate estimation a strong benzidine hydrochloride reagent which provides a large concentration of unprecipitated benzidine ions is favourable. A practical limit is however set to the strength of the benzidine reagent by the fact that strong reagents favour co-precipitation of benzidine hydrochloride and therefore lead to high results.

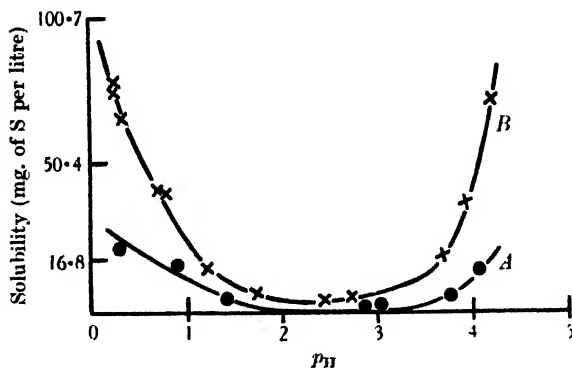


Fig. 1. Variation of solubility of benzidine sulphate with varying p_H .

Benzidine acetate was tried as a precipitating reagent but was unsatisfactory owing to the difficulty of attaining the optimum p_H save in presence of a large excess of acetic acid; substitution of chloroacetic acid as acidifying agent was of no assistance.

The effect of p_H on the precipitation of benzidine phosphate.

Solutions of KH_2PO_4 (Analytical Reagent, B.D.H.) were prepared. Rosenheim and Drummond's [1914] benzidine reagent (containing 2 g. base per litre and having p_H 1.94) was added. The mixture was kept for 10 min. after which the precipitated benzidine phosphate was filtered off on paper or on sintered glass filters. The p_H of the filtrate was determined electrometrically. The precipitate was titrated at the boiling-point with standard NaOH using phenolphthalein as indicator; calculations of the phosphate precipitated were made on the assumption that red coloration of the indicator represented approximately complete conversion of H_3PO_4 into Na_2HPO_4 .

The concentration of the phosphate solutions used varied between 1.3 and 0.45 mg. P/ml.; 1 mg./ml. is the concentration of P normally present in a 24-hour sample of human urine [Hawk (Bergeim), 1931]. Variations of p_H were obtained by prior addition of either acetate buffers or hydrochloric acid.

The results in Table IV show that some benzidine phosphate may be precipitated even when the p_H is as low as 1.4 (no. 12 of Table IV). Slight variations in p_H exercise a marked effect on the precipitation of benzidine phosphate, the percentage precipitated increasing rapidly as the p_H changes from 2 to 4. At p_H 2.75, which is optimum for the precipitation of benzidine sulphate, considerable amounts of benzidine phosphate are precipitated.

Table V shows the extent to which the presence of phosphate may interfere in the estimation of sulphate by the benzidine method. In these experiments a solution containing 2.961 g. Na_2SO_4 and 3.993 g. KH_2PO_4 was used; the concentrations of SO_4^{2-} and of PO_4^{3-} were thus about the same as in normal human 24-hour urine. To this solution benzidine hydrochloride reagent was added.

Table IV. *Effect of p_H on precipitation of benzidine phosphate from pure phosphate solutions.*

(Normal human 24-hour urine contains 1 mg. P per ml.)

No.	Vol. of KH_2PO_4 sol. ml.	Vol. of benz. reagent 2 g./l.	Phosphate mg. P		% pre-cipitated	p_H of filtrate
			Present	Found		
1	20	80	9.03	None	0	1.4
2	20	80	9.03	0.7	7.7	2.3
3	20	80	9.03	None	0	2.35
4	20	80	9.03	3.8	42	2.4
5	20	80	9.03	5.2	58	2.6
6	20	80	9.03	4.7	52	2.6
7	20	80	9.03	5.9	66	3.6
8	20	80	9.03	6.6	73	4.2
9	20	80	25.08	0.40	1.6	1.8
10	20	80	25.08	12.1	48.2	2.0
11	150	60	12.54	6.98	55.7	3.2
12	5	20	4.5	0.37	8.2	1.4
13	5	20	4.5	1.18	26.2	2.2
14	5	20	4.5	2.12	47.1	2.3
15	5	20	4.5	2.23	49.6	2.4

Table V. *Effect of the presence of phosphate on the estimation of sulphate.*

No.	Vol. of $Na_2SO_4 + KH_2PO_4$ sol. ml.	Vol. of water added ml.	Vol. of 2.5 N HCl added ml.	Vol. of benz. reagent added 8 g./l.	Sulphate mg. S		% error	Phosphate present in precipitate mg. P	p_H of filtrate
					Present	Found			
1	20	20	None	20	13.36	14.09	+ 5.4	0.48	1.9
2	20	20	..	20	13.36	14.09	+ 5.4	0.34	1.9
3	20	20	..	20	13.36	14.0	+ 4.5	0.40	1.9
4	20	20	..	20	13.36	13.96	+ 4.2	0.36	1.9
5	20	20	..	20	13.36	14.09	+ 5.4	0.48	1.9
6	20	20	..	20	13.36	14.20	+ 6.0		1.9
7	20	20	..	20	13.36	13.89	+ 3.7	Not analysed	1.9
8	20	18.4	1.6	20	13.36	13.11	- 1.9		1.1
9	20	18.4	1.6	20	13.36	12.96	- 3.0		1.1

The precipitate was filtered, washed and titrated in the usual way, the p_H of the filtrate being determined electrometrically.

In nos. 1-5 (Table V) the amount of benzidine phosphate precipitated was estimated quantitatively by the method of Tisdall [1922] after solution of the precipitate in excess of alkali and removal of the benzidine by extraction with ether.

As will be seen from Table V at p_H 1.9 enough benzidine phosphate was precipitated to cause an error of +4% to +6% in the sulphate estimation. In nos. 8 and 9, where an amount of HCl was added sufficient to reduce the p_H to 1.1, the sulphate recovery was 2-3% low owing to incomplete precipitation of benzidine sulphate.

Even at a p_H as low as 1.1 the precipitated benzidine sulphate was not free from contamination with benzidine phosphate as was shown by quantitative analysis of precipitates obtained at this p_H for phosphate.

In addition to p_H , time of standing before filtration and variations in temperature exercise marked effects on the precipitation of benzidine phosphate. Precipitation of benzidine sulphate is complete in 10 min. and is not affected by a longer time of standing (see also Rosenheim and Drummond [1914]), but the filtrates from a mixed benzidine phosphate-sulphate precipitate often deposited

benzidine phosphate on standing. The error in sulphate estimations due to contamination with benzidine phosphate was greatly increased if the reaction mixture was kept 24–48 hours before filtration. Variations in laboratory temperature were also found to affect the amount of benzidine phosphate precipitated. At lower temperatures larger amounts were precipitated.

Removal of phosphate.

Three different methods of phosphate removal were investigated, *viz.* those of Fiske [1921], Yoshimatsu [1926] and Hoffmann and Cardon [1935].

Hoffman and Cardon's method, originally designed for serum, in which the coagulation of a ferric hydroxide solution is made use of, was useless since the coagulum could not be washed free from sulphate [cf. Freundlich, 1928].

Both Fiske's method, in which phosphate is removed as MgNH_4PO_4 , and Yoshimatsu's method, in which phosphate is removed by means of uranyl acetate in the presence of acetate buffer at p_{H} 5, gave precipitates which were readily washed free of sulphate and filtrates which were free of phosphates when tested with strychnine molybdate. Using a dilution of 1 : 5 in removing phosphate and employing the benzidine hydrochloride reagent of Fiske, both the latter's and Yoshimatsu's method were found to give results concordant with those obtained by the method of Folin [1905], when applied to a solution of Na_2SO_4 and KH_2PO_4 (B.D.H. Analytical Reagents). These two methods were both tested on a 24-hour sample of human urine. The results are shown in Table VI. The p_{H} in these experiments was attained by making the solution, before addition of the benzidine reagent, just yellow to bromophenol blue by means of drops of N HCl.

Table VI. *Analysis of human urine (24-hour sample) by the benzidine method after removal of phosphate.*

Method of removal of phosphate	Vol. of original urine analysed ml.	Vol. after phosphate removal ml.	Titration 0.0172 N NaOH ml.	S found mg./l.	p_{H} of filtrate
MgNH_4PO_4	4	20	13.56	929	2.7
			13.62	932	2.5
			13.55	927	2.5
Uranyl acetate	4	20	13.53	927	2.8
			13.66	936	2.9
			13.66	936	3.1
		Wt. of BaSO_4 (g.)			
Phosphate not removed. Folin's gravimetric method	30	0.2012 0.2014	—	921.5	—

The method of Fiske, since it does not involve heating the urine (which is essential to the uranium method), seems to be preferable.

Effect of chlorides.

Fiske [1921] and Kahn and Lieboff [1928] state that the presence of NaCl increases the solubility of benzidine sulphate. Fiske showed that with a solution, of which the sulphate concentration was comparable with that of normal urine, analysis by Drummond's method might lead to 97 % precipitation of sulphate if the Cl : S ratio was 30 : 1 or to 91 % precipitation if the ratio were 60 : 1. Kahn and Lieboff report an error of –25 % when the Cl : S ratio is 90 : 1.

The Cl : S ratio in a 24-hour sample of normal human urine is 7 : 1 [Hawk (Bergeim), 1931]. Experiments were therefore performed in which sulphates were precipitated by benzidine hydrochloride from Na_2SO_4 solutions containing varying amounts of NaCl. The results are given in Table VII from which it will

Table VII. *Effect of chloride on precipitation of benzidine sulphate.*

No.	Vol. of Na_2SO_4 sol. analysed ml.	S present in experiment mg. S	Chloride present mg. Cl	Cl:S ratio	Vol. of benz. reagent 8 g./l. ml.	S found mg.
1	20	6.67	0	0	10	6.66
2	20	6.67	0	0	10	6.68
3	20	6.67	243	36.4	10	6.66
4	20	6.67	243	36.4	10	6.65
5	20	6.67	61	9.1	10	6.63
6	20	6.67	61	9.1	10	6.66
7	40	3.36	0	0	5	3.22
8	40	3.36	0	0	5	3.22
9	40	3.36	122	36.3	5	3.12
10	40	3.36	122	36.3	5	3.06
11	40	3.36	486	145	5	2.88
12	40	3.36	486	145	5	2.82

be seen that, when Cl^- and SO_4^{2-} are present in the concentrations found in normal human 24-hour urine, the results obtained are accurate within the limits of experimental error. When the sulphate solution is more dilute, however, chloride even in the amount present in normal urine prevents complete precipitation of benzidine sulphate.

SUMMARY.

1. In an aqueous medium benzidine sulphate has minimum solubility at p_{H} 2.75 ± 0.3 . In analysis it is therefore desirable to attain this p_{H} in the reaction mixture.
2. Benzidine phosphate is precipitated from solutions of potassium phosphate similar in concentration to a normal human 24-hour urine sample at p_{H} values as low as 1.4 and the amount of phosphate precipitated increases rapidly with p_{H} . In analysis of urine it is therefore desirable to remove phosphate. Either the method of Fiske [1921] or that of Yoshimatsu [1926] is suitable for this purpose.
3. The excess of benzidine left unprecipitated has an important effect in decreasing the solubility of benzidine sulphate. The supernatant fluid should contain about 1 g. benzidine per litre.
4. In sulphate solutions of concentration comparable with a normal human 24-hour urine sample, chloride in amounts much larger than physiologically normal does not interfere. In more dilute sulphate solutions chloride leads to low results.
5. A saturated solution of benzidine sulphate in water is a convenient and suitable liquid for washing the precipitated benzidine sulphate.

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LVII. THE ALLEGED PRESENCE OF DEHYDRO-ASCORBIC ACID IN BLOOD.

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SOME years ago Van Eekelen *et al.* [1933] found that the capacity of trichloro-acetic acid filtrates from the blood of man, rabbit, pig and guinea-pig for reducing indophenol was increased on treatment with hydrogen sulphide and further that on addition of vitamin C to blood *in vitro* it quickly passed into dehydroascorbic acid. As a consequence it has apparently become generally assumed that dehydroascorbic acid is present in blood with the implication that the latter is capable of dehydrogenating ascorbic acid.

In a previous communication [Kellie and Zilva, 1935] we had already shown that plasma, intact erythrocytes and leucocytes were unable to oxidise ascorbic acid *in vitro* and had further pointed out that the general evidence available was not only against the above assumption but, on the contrary, favoured the theory that the animal organism tended to reduce dehydroascorbic acid. We are now producing further experimental evidence showing that blood does not contain the reversibly oxidised form of the vitamin. The contrary view which some workers hold has, we believe, been responsible for a considerable confusion of thought in the interpretation of results obtained in this field of research.

EXPERIMENTAL.

The capacity of blood to reduce indophenol after treatment with hydrogen sulphide.

(a) *The influence of hydrogen sulphide on whole plasma.* The results presented in this connection are representative examples of a number of observations made by one of us (S. S. Z.) in the course of an investigation of wider scope. It was found that when ascorbic acid was added to whole plasma it could be accounted for with a good degree of accuracy by direct indophenol titration in acid solution even in concentrations as low as 1-2 mg. per 100 ml. There was nothing in plasma, free from haemoglobin, to interfere with the end-point. Failure to reduce the dye, therefore, indicated the absence of ascorbic acid from the plasma. Yet such plasmata were found to acquire the capacity of reducing indophenol to a very marked extent after treatment with hydrogen sulphide.

The procedure adopted in these experiments was as follows. The blood was collected in potassium oxalate solution (1 ml. 5 % potassium oxalate for 20 ml. blood) quickly centrifuged and the plasma carefully pipetted off. About 5 ml. of plasma containing one or two drops of octyl alcohol were placed in a glass bubbler of 15 ml. capacity and hydrogen sulphide passed for 5 min. The bubbler was then sealed and allowed to remain overnight. The hydrogen sulphide was removed by passing a stream of nitrogen through the solution for 24 hours. A dry lead acetate paper then held for 5 min. close to the fine orifice of the outlet tube invariably failed to stain appreciably, thus showing the absence of significant traces of volatile sulphides in solution. The p_H of the plasma fell from 7.4 to 6.8, while saturated with hydrogen sulphide, but regained its original value after the

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latter was displaced by the nitrogen. The titrations of the plasma samples were carried out at p_{H} 2.5–3.0 before and after treatment. The added indophenol was decolorised quickly by the plasma as it would have been if ascorbic acid were present.

The figures in Table I show that the amounts of indophenol decolorised by the hydrogen sulphide-treated plasma both of scorbutic guinea-pigs and of guinea-pigs kept on a mixed diet containing cabbage were of the same order.

Table I.

Wt. of animal g.	Scorbutic diet days	ml. N/1000 indophenol per ml. plasma		Wt. of animal g.	Diet	ml. N/1000 indophenol per ml. plasma	
		Before H_2S	After H_2S			Before H_2S	After H_2S
285	3	0.0	3.0	250	Mixed	0.0	5.1
275	5	0.0	2.2	260	"	0.0	5.2
280	7	0.0	5.2	280	"	0.0	4.0
285	8	0.0	5.2	280	"	0.0	4.2
295	11	0.0	3.0	410	"	0.0	8.6
300	13	0.0	4.2	420	"	0.0	4.7
360	13	0.0	5.0	575	"	0.0	6.3
385	14	0.0	2.7	580	"	0.0	2.3
372	15	0.0	4.0	580	"	0.0	4.6
395	15	0.0	5.8	635	"	0.0	5.3
210	22	0.0	4.5	650	"	0.0	2.7
270	22	0.0	4.1	750	"	0.0	3.3
620	23	0.0	4.2				
270	26	0.0	3.3			Average	4.7
		Average	4.0				

Table II.

Wt. of animal g.	Scorbutic diet days	Quantity injected (mg.)	Bled after min.	ml. N/1000 indophenol per ml. plasma	
				Before H_2S	After H_2S
315	7	100	5	8.9	4.0
310	7	100	10	6.3	3.3
285	10	100	15	2.2	3.3
300	8	100	120	0.0	6.3
300	10	100	120	0.0	4.9
				Average	4.4

Table II gives the values of the reducing capacities of the untreated and treated plasma of guinea-pigs which received ascorbic acid by intravenous injection. When high doses (100 mg.) are administered in this way ascorbic acid can be detected in the blood of the animals by titration with indophenol for about an hour afterwards. Yet the samples of blood, examined at various intervals following the injection, have shown reducing capacities after treatment with hydrogen sulphide no different from those obtained in the case of scorbutic guinea-pigs and animals which received cabbage daily.

Consequently serious doubts were expressed as to whether the reduction of indophenol by the treated plasma in a way so characteristic of ascorbic acid was really due to the vitamin. If this were so they should have contained 30–40 mg. per 100 ml. of plasma. It is difficult to conceive that guinea-pigs which subsisted for periods up to 26 days on a vitamin C-deficient diet, and some of these even in the premortal phase, should all have about the same concentration of the reversibly oxidised form of the vitamin in their blood as animals which received large quantities of the reduced form either *per os* or

by injection into the blood stream. This is the more striking since dehydroascorbic acid is as active antiscorbutically as ascorbic acid. Furthermore, it has been found that solutions of ascorbic acid will not remain at p_H 6.8-7.4 without serious loss during the period of hydrogen sulphide treatment as described above. Additional, and to our mind conclusive, proof will be given in a subsequent section that the reducing substance thus formed in the plasma is indeed not ascorbic acid.

(b) *The influence of hydrogen sulphide on trichloroacetic acid filtrates of plasma, erythrocytes and whole blood.* To the plasma obtained from the blood as previously described, an equal volume of 20% trichloroacetic acid was added. After centrifuging, the supernatant fluid was filtered and treated with calcium carbonate until no longer acid to Congo red. This partially neutralised solution was saturated with hydrogen sulphide and allowed to remain in a sealed glass bubbler for 12 hours. The hydrogen sulphide was then displaced by a current of nitrogen. Preliminary experiments have shown that the indophenol-reducing capacity acquired by the trichloroacetic acid filtrates from plasma after treatment with hydrogen sulphide varied firstly with the time allowed for the filtrate to decolorise the indicator and secondly with the thoroughness of the displacement by nitrogen of the hydrogen sulphide or other volatile substances capable of darkening dry lead acetate papers. In order to obtain a truer assessment of the character of the indophenol-reducing substance it was considered desirable to titrate the hydrogen sulphide-treated filtrates at two stages of the displacement and in each case to observe two end-points, the first the point at which 0.1 ml. $N/1000$ indophenol (dimethylaminobenzoinophenol) remained undecolorised after 10 sec. and the second that at which the same amount remained undecolorised after 30 sec. The first stage of the displacement was arbitrarily fixed at the point at which 5 min. were required for a black spot to be produced on a dry lead acetate paper held at the orifice of the bubbler. The solutions were then titrated again at a later stage when only a brown stain developed on the dry lead acetate papers after 15-min. exposure. These criteria were preferable to a time basis of comparison in the case of these experiments since they indicated more accurately corresponding states of the solutions.

The results (Table III) show that the highest figures were obtained after treatment with hydrogen sulphide when the 30-sec. end-point was used and when the extracts were titrated at the first stage of the passage of nitrogen. In the case of plasma E this figure reached an equivalent to 3.7 mg. of ascorbic acid per 100 ml. equivalent of plasma. When, on the other hand, the 10-sec. end-point was used, the equivalent fell to 0.9 mg. of ascorbic acid.¹ That this difference between the figures obtained with the two end-points was characteristic only of the reduced plasma filtrates and was not due to ascorbic acid is seen from the fact that in all the experiments in which authentic ascorbic acid was added to the original non-reducing plasma the trichloroacetic acid filtrates showed the same reducing values with both end-points.

Further examination of the results shows that even the reduction of indophenol by the samples of plasma observed when the 10-sec. end-point was employed could not be attributed to the presence of ascorbic acid. It will be seen, for instance, that when the passage of the nitrogen was prolonged to a stage at which the lead acetate paper reaction had become very weak, the indophenol reducing values of the solutions fell considerably. That the fall was not due to destruction of any formed ascorbic acid by the prolonged treatment is

¹ When 2:6-dichlorophenolindophenol is used the difference obtained between the two end-points is not so high.

Table III.

	Indophenol reduction, mg. equivalent ascorbic acid/100 ml. plasma			
	Plasma		Plasma + 2 mg. ascorbic acid/ 100 ml.	
	10 sec.	30 sec.	10 sec.	30 sec.
Plasma A.				
Before treatment with H_2S	0.0	0.0	2.1	2.1
After treatment with H_2S and displacement with nitrogen:				
(1) Blackening of dry $PbAc_2$ paper after 5 min.	0.0	1.1	2.5	3.0
(2) Brown staining of dry $PbAc_2$ paper after 15 min.	0.0	1.1	2.5	2.8
Plasma B.				
Before treatment with H_2S	0.0	0.0	2.1	2.1
After treatment with H_2S and displacement with nitrogen:				
(1) Blackening of dry $PbAc_2$ paper after 5 min.	0.9	2.1	5.3	5.5
(2) Brown staining of dry $PbAc_2$ paper after 15 min.	0.2	1.2	2.3	3.3
Plasma C.				
Before treatment with H_2S	0.0	0.0	2.3	2.3
After treatment with H_2S and displacement with nitrogen:				
(1) Blackening of dry $PbAc_2$ paper after 5 min.	0.7	2.5	3.7	6.2
(2) Brown staining of dry $PbAc_2$ paper after 15 min.	0.2	1.4	2.3	3.3
Plasma D.				
Before treatment with H_2S	0.0	0.0	2.3	2.3
After treatment with H_2S and displacement with nitrogen:				
(1) Blackening of dry $PbAc_2$ paper after 5 min.	0.9	3.3	3.3	5.6
(2) Brown staining of dry $PbAc_2$ paper after 15 min.	0.0	1.9	2.3	4.4
Plasma E.				
Before treatment with H_2S	0.0	0.0	2.1	2.1
After treatment with H_2S and displacement with nitrogen:				
(1) Blackening of dry $PbAc_2$ paper after 5 min.	0.9	3.7	3.7	5.1
(2) Brown staining of dry $PbAc_2$ paper after 15 min.	0.2	1.6	2.3	3.3

seen from the fact that in all the experiments the added ascorbic acid could be, considering the limitation of the method, satisfactorily accounted for at the second stage. This can be seen if the reducing values of the blank samples are subtracted from those obtained in the samples containing the added vitamin.

There still remained to be ascertained in this connection the behaviour of trichloroacetic acid extracts of erythrocytes and of whole blood after reduction with H_2S especially as in most if not all the experiments mentioned in the literature such extracts of whole blood were employed. The whole blood was extracted in the same way as the plasmata. In the case of the erythrocytes, on the other hand, a quantity of 20% trichloroacetic acid equal to that amount of the plasma removed by centrifuging from the blood was added. The titrations of the extracts were also carried out as above and at two similar stages. It may, however, be noted that in these experiments the nitrogen had to be passed for a longer time in order to reduce the darkening of the lead acetate papers to the required intensities. Only one end-point, namely, the inability of the solution to decolorise 0.10 ml. of a $N/1000$ solution of the indicator during 10 sec., was observed in these experiments. The results are given in Table IV and need hardly any comment. As in the case of the whole plasma a capacity for reducing indophenol, but only to a very much lower extent, was acquired by the trichloroacetic acid extracts in both cases, but this reducing property, unlike that of ascorbic acid, gradually diminished during the passage of nitrogen until it totally vanished at the second stage of the displacement.

Table IV.

	Indophenol reduction, mg. equivalent ascorbic acid/100 ml. blood							
	Corpuscles				Corpuscles + 1.5 mg. ascorbic acid/100 ml. blood			
	0.0	0.0	0.0	0.0	1.5	1.5	1.4	1.4
Before treatment with H ₂ S								
After treatment with H ₂ S and displacement with nitrogen:								
Brown staining of dry PbAc ₂ paper after 5 min.	0.7	0.6	0.6	0.6	1.7	1.8	1.7	1.7
Brown staining of dry PbAc ₂ paper after 15 min.	0.0	0.0	0.0	0.0	1.7	1.5	1.5	1.4
	Whole blood				Whole blood + 1.5 mg. ascorbic acid/100 ml. blood			
	0.0		0.0		1.4		1.5	
Before treatment with H ₂ S								
After treatment with H ₂ S and displacement with nitrogen:								
Brown staining of dry PbAc ₂ paper after 5 min.	0.9	0.7			2.5	1.9		
Brown staining of dry PbAc ₂ paper after 15 min.	0.0	0.0			1.4	1.5		

Taking the above points into consideration it is concluded that the very low capacity for reducing indophenol by trichloroacetic acid filtrates of plasmata, erythrocytes and whole blood treated with hydrogen sulphide cannot be due to the presence of quantities of ascorbic acid capable of detection by this method.

(c) *The possible precursors of the indophenol-reducing substances formed on treatment of whole plasma with hydrogen sulphide.* This problem has not been studied in detail as it was considered to be too far removed from the aim of the general scope of the work which prompted the present research. It may, nevertheless, be of interest to describe here experiments which, although not complete in themselves, are likely to shed some light on the subject. It was found that when whole plasma was precipitated with absolute alcohol and the alcoholic filtrate treated with hydrogen sulphide according to the above technique it acquired a much higher reducing capacity for indophenol than the original plasma. On the other hand, after further precipitation with cadmium chloride the alcoholic filtrate did not acquire this property at all.

15 ml. of absolute alcohol were gradually added to 5 ml. of plasma from guinea-pigs on a mixed diet. After removing the precipitate by centrifuging, the supernatant liquid was divided into two equal parts. One was reduced with hydrogen sulphide without further treatment, the other was precipitated with alcoholic cadmium chloride (0.1 ml. 2.5 % CdCl₂ per 1 ml. of solution). After standing for 15 min. the precipitate was removed on the centrifuge and the

Table V.

Alcohol filtrate before precipitation with CdCl ₂ , mg. ascorbic acid equivalent per 100 ml. plasma		Alcohol filtrate after precipitation with CdCl ₂ , mg. ascorbic acid equivalent per 100 ml. plasma	
Before treatment with H ₂ S	After treatment with H ₂ S	Before treatment with H ₂ S	After treatment with H ₂ S
0.0	87.5	0.0	0.0
0.0	122.8	0.0	0.0
0.0	70.0	0.0	0.0

resulting supernatant alcoholic solution was also submitted to hydrogen sulphide treatment. In Table V the reducing values obtained in these experiments are expressed for convenience only as ascorbic acid, which does not, however, imply that the reduction was due to ascorbic acid. All the evidence is conclusively against this assumption.

Spectrographic examination of plasma before and after treatment with hydrogen sulphide.

An attempt was next made to ascertain spectrographically whether ascorbic acid was present in plasma reduced with hydrogen sulphide. For this purpose both non-reducing normal samples of plasma and reduced samples of plasma each alone and also with the addition of authentic ascorbic acid (20 mg. and 2 mg. of ascorbic acid per 100 ml. of plasma respectively) were examined.

The samples free from haemoglobin were obtained from the blood by the procedure described above. The non-reducing normal and reduced plasmata were then precipitated with freshly distilled absolute alcohol which was added slowly with constant stirring until a final concentration of 80 % alcohol was reached. The corresponding preparations containing ascorbic acid were obtained by the addition of (a) 2 ml. of 98 % alcohol containing 0.4 mg. ascorbic acid, (b) 2 ml. of 98 % alcohol containing 0.04 mg. ascorbic acid respectively, to 2 ml. plasma, before precipitating the proteins with alcohol. The precipitates were removed on the centrifuge. 5 ml. of each centrifugate were diluted with 4 ml. of freshly distilled absolute alcohol and acidified with 1 ml. of 0.2 *N* HCl, thus maintaining the concentration of alcohol at 80 % in the final solutions. These solutions were compared in the spectrograph with blanks containing 0.02 *N* HCl in 80 % alcohol. The solutions prepared from plasma containing 20 mg. ascorbic acid per 100 ml. were examined in 1 cm. absorption tubes, whilst in the case of the more dilute solutions 4 cm. tubes were found to be more convenient. A Hilger quartz spectrograph with sector photometer and Ilford Q 3 plates were employed. The source of light consisted of a condensed oscillatory discharge between electrodes composed of a mixture of cadmium and iron. These electrodes gave more intense spectral lines in the lower region of the spectrum than the normal tungsten steel.

Figs. 1 and 2 are self explanatory. There is no suggestion of any significant difference in the absorption in the region of 245 $m\mu$ between plasma treated with hydrogen sulphide and the normal non-reducing plasma. On the other hand, on the addition of 20 mg. of ascorbic acid to 100 ml. of the plasma, a concentration well below the calculated average obtained from the indophenol-reducing figures of all the samples of the treated whole plasma, well-marked characteristic peaks at 245 $m\mu$ were obtained in both the reduced and non-reduced media. Furthermore, definite absorption in this region was established with a concentration as low as 2 mg. of ascorbic acid per 100 ml. of plasma.

DISCUSSION.

The entire evidence obtained in this investigation does not lend support to the view that blood of normal guinea-pigs contains demonstrable quantities of dehydroascorbic acid. It is true that it was found that whole plasma free from ascorbic acid could on treatment with hydrogen sulphide acquire the capacity of reducing indophenol to a very marked extent, but this was shown at the same time not to be due to the formation of ascorbic acid. The examination of trichloroacetic acid filtrates of the blood from normal guinea-pigs has revealed that they too can acquire by the same means a similar indophenol-reducing capacity, although to a very much less extent than the whole plasma. The evidence of

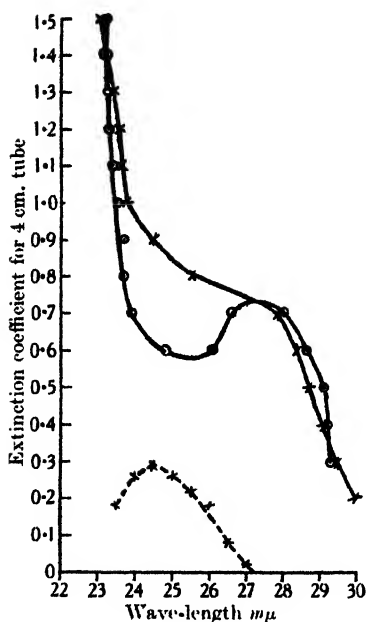


Fig. 1 A.

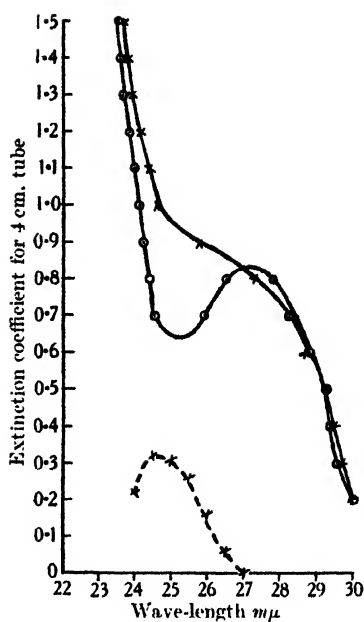


Fig. 1 B.

Fig. 1. A, $\times-\times$ Plasma of mixed diet pig + 2 mg. ascorbic acid per 100 ml.; $\circ-\circ$ plasma of mixed diet pig; $\times---\times$ ascorbic acid curve by subtraction. B, $\times-\times$ Plasma of mixed diet pig treated with H_2S + 2 mg. ascorbic acid per 100 ml.; $\circ-\circ$ plasma of mixed diet pig treated with H_2S ; $\times---\times$ ascorbic acid curve by subtraction.

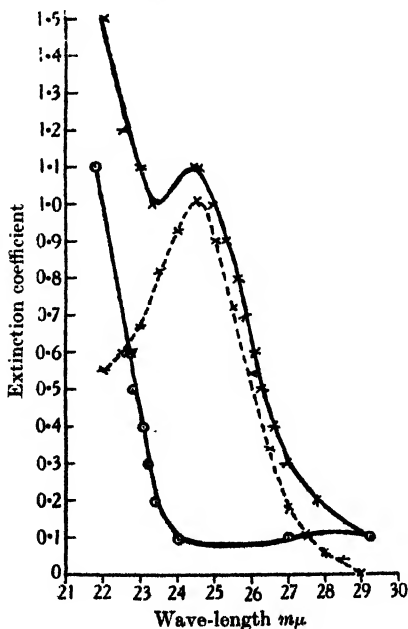


Fig. 2 A.

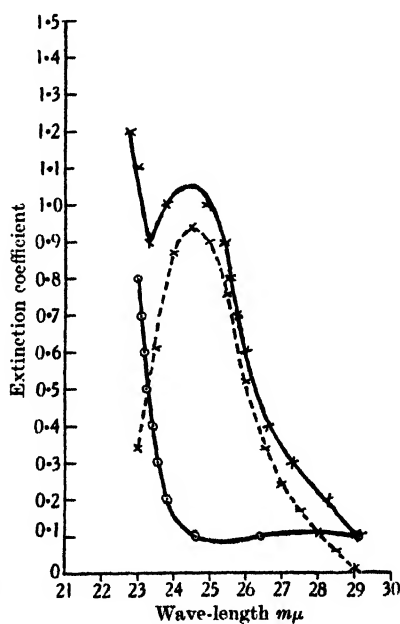


Fig. 2 B.

Fig. 2. A, $\times-\times$ Plasma of mixed diet pig + 20 mg. ascorbic acid per 100 ml.; $\circ-\circ$ plasma of mixed diet pig; $\times---\times$ ascorbic acid curve by subtraction. B, $\times-\times$ Plasma of mixed diet pig treated with H_2S + 20 mg. ascorbic acid per 100 ml.; $\circ-\circ$ plasma of mixed diet pig treated with H_2S ; $\times---\times$ ascorbic acid curve by subtraction.

this paper shows, however, that this acquired reducing capacity is due essentially to an artefact and not, as is assumed by some workers, to the reduction of dehydroascorbic acid.

The statement of Van Eekelen *et al.* that vitamin C added to blood *in vitro* quickly passes into the reversible form must now be considered. These workers do not give any experimental details, but their observation can be readily explained on our established facts. We have already shown [Kellie and Zilva, 1935] that in the presence of laked erythrocytes ascorbic acid was in the first place oxidised, most probably by oxyhaemoglobin, to an extent which was proportional to the amount of haemolysed cells added. After this initial reaction the disorganised corpuscles exercised a protective action against the oxidation of further amounts of ascorbic acid. We, therefore, assume that the formation of dehydroascorbic acid observed by Van Eekelen *et al.* was due most probably to a partial laking of the blood employed in their experiments. It is essential to note that at the same time we found that intact erythrocytes did not manifest this initial oxidising property, but were, on the contrary, capable of preventing oxidation. The present investigation throws further light on the matter. The red corpuscles of the blood of guinea-pigs on a mixed diet with cabbage *ad lib.* do not contain any dehydroascorbic acid nor in fact do any of the other constituents of the blood. In view of these observations it is very difficult to reconcile the results of our experiments with the assumption that blood in which the erythrocytes are intact can dehydrogenate and store ascorbic acid in quantities capable of detection by our present methods.

SUMMARY.

Whole plasma of guinea-pigs subsisting on a diet containing cabbage *ad lib.*, of guinea-pigs injected intravenously with high doses of ascorbic acid about an hour before bleeding and of scorbutic guinea-pigs acquires the property of reducing indophenol after treatment with hydrogen sulphide to about the same extent. Calculated in terms of ascorbic acid, these would contain about 38 mg. of the vitamin per 100 ml. of plasma. It is shown, however, that this acquired reducing capacity is not due to ascorbic acid.

Trichloroacetic acid extracts of whole blood, plasma and erythrocytes also reduce indophenol after treatment with hydrogen sulphide to a very much smaller extent, *i.e.* to an equivalent of 1–3 mg. per 100 ml. of blood. In this case also the acquired reducing capacity is not due to ascorbic acid but is shown to be an artefact. Unlike the reduction of the indicator by ascorbic acid of similar concentration it is influenced by the thoroughness of the displacement by nitrogen of the hydrogen sulphide and other volatile substances capable of staining dry lead acetate paper and by the time taken to decolorise the indicator (dimethylaminobenzoinndophenol) by the solutions within 10–30 sec.

This evidence lends further support to the authors' assertion that blood with its erythrocytes intact is incapable of dehydrogenating ascorbic acid *in vivo* and storing it in quantities capable of being detected by the existing methods and that there is a tendency of the animal organism to keep the vitamin in a reduced stage.

One of us (A. E. K.) is indebted to the Medical Research Council for a whole time grant.

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LVIII. THE PREPARATION OF HEPTAACETYL-*dl*-GALACTOSE BY THE ACETOLYSIS OF AGAR.

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AGAR is more resistant to acetylation than many other polysaccharides and it can be recovered unchanged after treatment by the gentle methods of acetylation that have been described recently. Treatment with pyridine and acetic anhydride under various conditions or with acetic anhydride in the presence of SO_2 and Cl_2 , HCl , KCNS or ZnCl_2 are all alike ineffective, whereas the original acetylation method of Franchimont [1879] works well with this material. Nothing is gained by the use of perchloric rather than sulphuric acid as a catalyst.

With sulphuric acid as catalyst the rate and extent of the action depend on the temperature and acid concentration; with 1 volume of sulphuric acid in 6 or 7 of acetic anhydride there is little action at room temperature even in 3 days, whilst about 80 % of the agar dissolves in 2 days at 38° and in 2 hours at 55° . At higher temperatures all the agar dissolves but there is very serious blackening of the solution and no water-insoluble products can be isolated afterwards. The agar that remains unattacked after 2 days at 38° or 2 hours at 55° has the properties of untreated agar; it will make a gel in the usual way and when acetylated again under the same conditions it dissolves and gives similar acetylation products. From this point of view therefore agar is relatively homogeneous.

From the products of the acetolysis of agar a crystalline substance was isolated which proved to be heptaacetyl-*dl*-galactose; a method for the synthesis of this substance has meanwhile been published by Micheel *et al.* [1935].

The diacetyl derivatives of most of the common aldehydes have been prepared by the direct acetylation of the aldehydes under conditions similar to those used here with agar; attempts were therefore made to prepare heptaacetyl-*d*-galactose by acetylation of galactofuranose and galactopyranose derivatives. These attempts were unsuccessful but heptaacetyl-*d*-galactose could readily be isolated from the products of acetylation of aldehydogalactose derivatives. It appears therefore that under the conditions of acetylation the oxygen-containing ring in an ordinary galactose derivative is not opened and that it is necessary by some other means to get the sugar into the open chain form before acetylation of the aldehyde group can take place. The acetylation of some sugars in this way are described in the following paper. The fact that a heptaacetyl sugar can be obtained from agar may be adduced as evidence that in it the sugar exists, in part at any rate, in the aldehyde form. It is interesting in this connection to note that it has proved impossible to isolate heptaacetyl-galactose from the acetylation products of galactose methylphenylhydrazones although Wolfrom and Christman [1931] have brought forward evidence that this is a real hydrazone derived from aldehydogalactose and has not, like some other supposed hydrazones, a ring structure. It may be relevant to mention in this connection that galactose methylphenylhydrazone gives a very small yield of mucic acid on oxidation with nitric acid.

EXPERIMENTAL.

Heptaacetyl-dl-galactose.

Commercial powdered agar contains up to 20 % of water; this can be removed by heating the agar on a boiling water-bath *in vacuo*. Shredded agar is less easy to dry and it is not recommended as material for acetylation because, presumably on account of the smallness of the exposed surface, it is more slowly dissolved by the acetylation mixture.

To 10 g. of dried agar an ice-cold mixture of 40 ml. acetic anhydride and 6 ml. conc. H_2SO_4 is added. The mixture is shaken well and left at room temperature for an hour or two before being put in an incubator at 38°. The action is exothermic but no precautions are necessary when working on this scale: if larger quantities are used a thermometer should be kept in the acetylation mixture. The temperature should not be allowed to exceed 45° or the action may become violent. The mixture soon goes nearly black, it is shaken occasionally and taken out and cooled after about 44 hours. When cold it is rather viscous but it will filter through a sintered glass funnel. The residue of unacetylated agar is washed with 10–20 ml. of acetic acid. If dried it weighs 1–2 g. and, although somewhat discoloured, it has the properties of untreated agar, *i.e.* it will form a rigid jelly and when acetylated gives heptaacetyl-*dl*-galactose in the usual yield.

The filtrate and washings are poured into 300 ml. of water containing 35 g. of hydrated sodium acetate and some ice. After standing with occasional stirring for 2–3 hours the brown fluid and putty-like solid are distilled together to dryness *in vacuo*. Some water is added and the mixture is again distilled to remove the rest of the acetic acid.

50 ml. of water and 50 ml. of chloroform are added and the flask shaken until all the lumps of sodium sulphate and acetylation products have dissolved, the chloroform layer being then removed in a separating funnel. With the help of a strong beam of light the interface between the two dark-coloured layers can be fairly easily seen. The chloroform layer is washed once again with water and distilled to dryness *in vacuo*. 30–40 ml. of hot water are added and the gum is shaken into an emulsion. On cooling it resolidifies and the water can be poured off. The gum is dissolved in 30–40 ml. of hot alcohol and partially decolorised with charcoal; it is generally impossible to remove all the colour at this stage. The solution may also be decolorised by addition of half a volume of ether to the warm alcoholic solution. A brown flocculent precipitate separates which must be filtered off quickly for the heptaacetyl-*dl*-galactose is apt to precipitate soon. The latter will crystallise from the filtrate in a few hours, generally without seeding, in the form of a felt of needles sometimes embedded in jelly. After recrystallisation the yield is 1–2 g.

The melting-point is very much affected by impurities and after several recrystallisations and thorough drying it reaches 132°. Recrystallisation is most easily carried out from 30 parts of methyl or ethyl alcohol or from 150 parts of boiling water. In many other solvents the compound is inconveniently soluble and from pyridine, benzene, acetone and *cyclohexane* it retains solvent obstinately; after drying *in vacuo* at room temperature the solid may retain 1–2 % of solvent for several days but the amount is always lower than a molecular proportion. The solvent can be driven off by heating *in vacuo* to 100° or by boiling with water. In some solvents, benzene for example, the solvated form is less soluble than the solvent-free; the latter therefore dissolves quickly in a small amount of solvent and then, after a few minutes, a precipitate of the

solvated form separates. This property somewhat limits the choice of solvent for measuring the depression of freezing point.

The optical rotation of a pure sample in 10 % solution in chloroform was $<0.02^\circ$ in a 2 dm. tube. Since some galactose derivatives are known which have very low specific rotations [Wolfson, 1930; Levene and Meyer, 1927], a sample was hydrolysed by refluxing for 15 min. with N H_2SO_4 and the *dl*-galactose isolated by removing the sulphuric acid with baryta, concentrating and crystallising the sugar by the addition of alcohol. The yield was 80 % and the product contained 98 % of reducing sugar, had no measurable optical rotation and gave on oxidation with nitric acid as much mucic acid as the same weight of *d*-galactose. Furthermore the mother-liquor, on oxidation with nitric acid gave more mucic acid. This showed that *dl*-galactose and probably this alone occurred in the acetylated material.

When hydrolysed with $5N$ H_2SO_4 and then distilled with the continuous addition of water an amount of titratable acid corresponding to an acetyl content of 61–62 % was found in the distillate. These distillates were free from both carbon dioxide and formic acid and it was therefore necessary to assume that the substance did in fact contain more than one acetyl group for each carbon atom. The synthesis of the corresponding derivative of *d*-galactose confirmed the possibility of the existence of orthodiacetyl derivatives of the sugars. If these acetylated sugars are saponified by heating with alkali before distillation, large and variable amounts of carbon dioxide are formed: acid hydrolysis is therefore preferable.

The sugar content whether measured by the total carbohydrate method of Tillmans and Philippi [1929] or by the method of Hagedorn and Jensen was 36 %; in each case the ester was hydrolysed in the course of the estimation. When oxidised with nitric acid 509 mg. gave 133 mg. of mucic acid. This value is that to be expected, from Van der Haar's [1917] tables, for a heptaacetyl-galactose.

A 4 % solution (by volume) in acetic acid gave F.P. depression of 0.30° and a 4 % solution (by volume) in bromoform a depression of 0.40° . These correspond to mol. wt. 486 and 496 respectively. (Found (Weiler): C, 49.04; H, 5.85; acetyl, 62.3 %. $C_{20}H_{28}O_{14}$ with 7 acetyl groups requires: C, 48.76; H, 5.73; acetyl, 61.2 %.)

The analytical figures are a very unsatisfactory index of purity, since all likely contaminants would have a similar percentage composition. The melting point is better but the optical rotation is the best criterion. The uncrystallisable acetylated agar that remains in the alcoholic solution from which heptaacetyl-*dl*-galactose has been isolated can be obtained as a pale yellow powder that gradually softens to a gum having $[\alpha]_D$ about $+24^\circ$ in chloroform. The last trace of optical activity can always be removed from heptaacetyl-*dl*-galactose by crystallisation from acetone but the product obtained in this way requires intensive drying to remove the acetone.

Michael *et al.* [1935] prepared heptaacetyl-*dl*-galactose by acetylating tetra-acetylaldehyde-*d*-galactose-6-iodohydrin and explain the loss of optical activity by postulating the intermediate formation of a symmetrical cyclohexane derivative which then breaks down to give a *dl*-galactose derivative. The isolation of a derivative of *dl*-galactose cannot therefore be taken as conclusive proof of its preexistence in a complex molecule and it becomes necessary to obtain supplementary evidence.

There is nothing intrinsically improbable in the occurrence of *dl*-galactose in agar for it is already known as a constituent of Chagual gum [Winterstein,

1898] nori [Oshima and Tollens, 1901] and quince gum [von Lippmann, 1922] whilst *l*-galactose has been found in flax seed mucilage by Anderson [1933]. Hydrolysis for 1 hour with $N/10$ H_2SO_4 at 100° is sufficient to liberate 50 % of the reducing groups of agar as estimated by the Hagedorn-Jensen method; at this stage no free galactose has been liberated and the hydrolysate is neither fermented by galactose-trained yeast nor will it give a hydrazone with methylphenylhydrazine. Hydrolysis for 1 hour with N H_2SO_4 on the other hand gives a maximum yield of methylphenylhydrazone and a maximum evolution of carbon dioxide with galactose-trained yeast, the yields in each case corresponding to a galactose content, in terms of dry agar, of 28–30 %.

Lüdtke [1929] has isolated galactose methylphenylhydrazone from hydrolysed agar and I have followed his method in part: it is however unwise to heat the mixture of neutral hydrolysate, alcohol and methylphenylhydrazine to 37° as he does because this leads to the formation of a tarry material besides the hydrazone. The condensation is complete after 10–12 hours at room temperature. This hydrazone is decomposed with acetaldehyde by the method of Collatz and Neuberg [1932] and the sugar solution is allowed to crystallise with the addition of alcohol. The first crops of crystals are pure *d*-galactose but the later fractions crystallise with more difficulty and have a low optical rotation although they still contain 100 % of sugar. These later fractions and the mother-liquor are fermented with galactose-trained yeast for 3–5 days. Pure *l*-galactose can now be isolated by filtering off the yeast and adding alcohol to the concentrated filtrate. From 25 g. of agar only 200 mg. of *l*-galactose have so far been isolated in this way, corresponding to a yield of 1.6 % of *dl*-galactose. The yield of heptaacetyl-*dl*-galactose on the other hand may be as high as 20 %, corresponding to 7.2 % of *dl*-galactose. This discrepancy is serious and it cannot be claimed that in this case none of the *dl*-galactose has arisen by secondary actions during the acetylation. It will however be shown later that acetylation of hexoses is not generally accompanied by such a process.

The uncrystallisable gum from an acetylation carried out in this way has an acetyl content of 55–57 % and contains about half the carbohydrate of the agar used. The proportion of galactose in this acetylated agar is larger than in the original agar; there is little or no destruction of the galactose under these conditions and the loss of total carbohydrate on acetylation is mainly at the expense of the unknown constituents which make up 70 % of the molecule.

Agar which has been slightly hydrolysed, *e.g.* by boiling for 40 min. with $N/10$ H_2SO_4 , can be acetylated very much more easily than intact agar. Under these conditions of hydrolysis no free galactose is liberated and the hydrolysate, after acetylation with 1:20 H_2SO_4 -acetic anhydride for 24 hours, seems to contain no heptaacetyl-*dl*-galactose. If however this acetylated agar is subjected to further acetylation, heptaacetyl-*dl*-galactose can be isolated in the usual way and in the usual yield. Similarly, if the small amounts of acetylagar that can be obtained by treating agar for a short time or at a low temperature with the usual acetylation mixture are subjected to more vigorous acetylation, the heptaacetyl derivative can be isolated from them.

Agar which has been completely hydrolysed, *e.g.* by boiling for 1–2 hours with N H_2SO_4 , can also be easily acetylated but in this case no heptaacetyl-*dl*-galactose can be isolated. It is easy however to isolate α -pentaacetyl-*dl*-galactose and α -pentaacetyl-*d*-galactose by the same technique. These can be separated by fractional crystallisation from alcohol, the former being the less soluble.

Pentaacetyl-*dl*-galactose was identified by its acetyl content, 55.4 %, by the fact that it gave the expected yield of mucic acid after oxidation with nitric

acid and by its optical inactivity even after hydrolysis. It was presumably in the α -form since Micheel *et al.* [1935] give 111° as the melting point of the corresponding β -derivative whereas the substance prepared from agar melts at 144° . Furthermore it has been made by a method which, when used with *d*-galactose, gives rise to the α -pentaacetate.

These results are compatible with the hypothesis that in agar at least the *dl*-galactose occurs in a form other than the usual furanose or pyranose ring and that the heptaacetyl derivative results from preparations in which acetylation precedes complete hydrolysis. If the sequence is inverted or if the agar is subjected to such mild acetylation that complete hydrolysis does not ensue, heptaacetylgalactose cannot be isolated, in the first case because the sugar has cyclised and can only undergo pentaacetylation and in the second case because it is still attached to the other constituents of the polysaccharide. In the second case it can still be liberated by acetolysis. Further evidence that in agar the galactose occurs in an open-chain form will be presented in a later publication.

SUMMARY.

Heptaacetyl-*dl*-galactose is the only crystalline substance that can be isolated from the acetolysis products of agar. By the acetylation of hydrolysed agar α -pentaacetyl-*d*-galactose and α -pentaacetyl-*dl*-galactose can be prepared. The isolation of *l*-galactose from hydrolysed agar is described.

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LIX. THE HYPERACETYLATION OF ALDOSES.

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MICHEEL *et al.* [1935] have described the synthesis of heptaacetyl-*dl*-galactose and of heptaacetyl-*d*-galactose; this is the first instance of the diacetylation of the aldehyde group in a sugar. In the preceding paper I have described the isolation of *dl*-galactose from agar as the heptaacetyl derivative. The method of synthesis used by Micheel *et al.* is rather laborious; since sugar derivatives of this type are of interest for a number of reasons, a simpler and apparently general method for their preparation has been worked out. The diethylmercaptal or the acetylated diethylmercaptal of the sugar has been used in each case but it is probable that any other derivative of the aldehydic form of the sugar would do instead provided that it could break down under the conditions of hyperacetylation to give the free aldehyde.

Six hyperacetylated sugars have been made and all but one crystallise very readily. The small optical rotation and the readiness with which the melting points are affected by impurities diminish their usefulness for characterisation but the ease with which they can be crystallised even from a very crude solution suggests that they may prove useful for the separation of sugars.

Heptaacetyl-d-galactose.

Unsuccessful attempts were made to prepare heptaacetyl-*d*-galactose from *d*-galactose, *d*-galactosemethylphenylhydrazone, β -pentaacetyl-*d*-galactopyranose and β -pentaacetyl-*d*-galactofuranose [Schlubach and Prockownik, 1930] by acetylation under the conditions used with agar. In the first case α -pentaacetyl-*d*-galactopyranose (M.P. 96° , $[\alpha]_D + 103^\circ$ and acetyl content 55%) was isolated and in the last some pentaacetylgalactofuranose was recovered unchanged. In the other two cases no crystalline material could be isolated.

Pentaacetylaldehyde-*d*-galactose and its diethylmercaptal were therefore prepared [Wolfrom, 1930] and these substances, together with galactose diethylmercaptal were found to give heptaacetyl-*d*-galactose on acetylation. There is considerable destruction and the yield is always low but it can be improved by using a shorter time of incubation and less sulphuric acid than is possible in the case of agar. If 2 g. of pentaacetyl-*d*-galactose diethylmercaptal are suspended in an ice-cold mixture of 20 ml. acetic anhydride and 1 ml. conc. H_2SO_4 , 0.7 g. of the heptaacetate can be isolated after 24 hours at 37° by the method used for the isolation of heptaacetyl-*dl*-galactose.

Large well-formed crystals separate when heptaacetyl-*d*-galactose is crystallised from alcohol but these contain alcohol and have a low M.P. ($85-90^\circ$). This alcohol is not readily removed in a desiccator. Small crystals are obtained from water or methyl alcohol and needles up to 1 cm. long are formed by the aggregation of an emulsion when heptaacetylgalactose is dissolved in 20 parts of boiling 25% methyl alcohol and then allowed to crystallise at 37° . After drying at room temperature *in vacuo* it melts at 106° and has $[\alpha]_D + 4.0^\circ$ in chloroform solution. (Found: C, 48.76; H, 5.80; acetyl 62.2%. Calculated: C, 48.76; H, 5.73; acetyl, 61.2%.)

When compared with galactose this substance is found to contain 36 % of reducing sugar by the Hagedorn-Jensen method and after acid hydrolysis galactose can be isolated in the usual way. This galactose, at equilibrium, had $[\alpha]_D^{18} + 80.3^\circ$ in water.

Heptaacetyl-d-mannose.

The pentaacetate of *d*-mannose diethylmercaptal has not apparently been made before. Mannose mercaptal [Levene and Meyer, 1927] was acetylated in the usual way with pyridine and acetic anhydride at room temperature. The oil which separated when the acetylation mixture was poured into water crystallised without much difficulty. From aqueous alcohol it crystallised as clumps of stout prisms, frequently pointed at one end; m.p. $51-52^\circ$; $[\alpha]_D^{17}$ in chloroform $+31.2^\circ$. Yield 80-90 %.

2 g. of this product gave 1.4 g. of heptaacetyl-*d*-mannose when acetylated for 24 hours at 38° with a mixture of 20 ml. acetic anhydride and 1 ml. H_2SO_4 . The substance crystallised in stout needles from dilute alcohol and melted, when thoroughly dried, at 122° . In chloroform solution $[\alpha]_D$ is $+0.4^\circ$ and, as in other cases, the mannose isolated after hydrolysis had the normal rotation. (Found: C, 49.05; H, 5.93; acetyl, 62.0 %. Calculated for $\text{C}_{18}\text{H}_{28}\text{O}_{14}$: C, 48.76; H, 5.73; acetyl, 61.2 %.)

Heptaacetyl-d-glucose.

Pentaacetyl-*d*-glucose diethylmercaptal [Wolfrom, 1929] can be acetylated under exactly the same conditions as the corresponding galactose derivative and, by the same process of isolation, gives rise to a somewhat larger amount of heptaacetylglucose, e.g. from 5 g. of non-crystalline pentaacetylglucose mercaptal 2.5 g. of heptaacetylglucose can be prepared. It can be recrystallised from methyl alcohol, ethyl alcohol or water but it is difficult to remove every trace of solvent from the crystals. m.p. $121-2^\circ$; $[\alpha]_D^{19} + 7.9^\circ$ in chloroform. Glucose having the normal optical rotation was isolated from this after acid hydrolysis. (Found: C, 49.34; H, 5.94; acetyl, 63.0 %. Calculated: C, 48.76; H, 5.73; acetyl, 61.2 %.)

Hexaacetyl-l-arabinose.

Tetraacetyl-*l*-arabinose diethylmercaptal (2 g.) [Wolfrom and Newlin, 1930] when acetylated in the usual way with 1 part H_2SO_4 to 20 of acetic anhydride gave 0.93 g. of the hexaacetate. This crystallises from dilute alcohol as sheaves of fine needles; m.p. 91° ; $[\alpha]_D^{17} - 27.7^\circ$ in chloroform. (Found: C, 48.95; H, 5.72; acetyl, 62.1 %. Calculated for $\text{C}_{17}\text{H}_{24}\text{O}_{12}$: C, 48.56; H, 5.76; acetyl, 61.4 %.)

Hexaacetyl-l-rhamnose.

l-Rhamnose diethylmercaptal [Fischer, 1894] was acetylated with pyridine and acetic anhydride and the product recrystallised from dilute alcohol. It crystallises in thin hexagonal plates; m.p. 60° ; $[\alpha]_D^{15} + 39.5^\circ$ in chloroform.

When acetylated with acetic anhydride and sulphuric acid this material gives a 67 % yield of hexaacetyl-*l*-rhamnose. This crystallises as clusters of fragile plates from aqueous methyl alcohol; m.p. $72-3^\circ$; $[\alpha]_D^{17} - 7.5^\circ$ in methyl alcohol. (Found: C, 49.92; H, 6.05; acetyl, 59.4 %. Calculated for $\text{C}_{18}\text{H}_{26}\text{O}_{12}$: C, 49.75; H, 6.03; acetyl, 59.44 %.)

Hexaacetyl-d-xylose.

Hexaacetyl-*d*-xylose has not yet been obtained crystalline but a pale yellow gum with the properties that would be expected for such a substance can be

isolated by the usual method from the products of the acetylation of tetraacetyl-*d*-xylose diethylmercaptal [Wolf from *et al.* 1931]. This gum contains 59 % of acetyl and in chloroform solution has $[\alpha]_D^{17} + 4.0^\circ$.

SUMMARY.

By the acetylation of pentaacetyl-*d*-galactose diethylmercaptal with acetic anhydride in the presence of sulphuric acid, heptaacetyl-*dl*-galactose has been prepared.

By the same method heptaacetyl derivatives of *d*-glucose and *d*-mannose and hexaacetyl derivatives of *l*-arabinose, *l*-rhamnose and *d*-xylose have been prepared for the first time.

The preparation and properties of pentaacetyl-*d*-mannose diethylmercaptal, and tetraacetyl-*l*-rhamnose diethylmercaptal are described.

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LX. INVESTIGATIONS ON THE ROOT NODULE BACTERIA OF LEGUMINOUS PLANTS.

XVIII. BREAKDOWN OF PROTEINS BY THE ROOT NODULE BACTERIA.

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THE literature of the root nodule bacteria contains very little information regarding the ability of these organisms to decompose proteins. Several investigators have shown that the nodule bacteria slowly liquefy gelatin. However, this slow liquefaction takes place only in older cultures so that the nodule bacteria cannot be classed among the gelatin-liquefying bacteria proper. No further data are available concerning the changes in gelatin caused by these organisms.

The behaviour of the nodule bacteria towards milk has been studied by several workers. Löhnis and Hansen [1921] showed that these bacteria can be divided into two groups according to the changes they produce in milk. The organisms of the first group form a serum zone in milk, whilst those of the second group do not. The first group includes the nodule organisms from most cultivated legumes. However, even the bacteria forming a serum zone effect only very slight changes in milk proteins. Thus, for instance, Fred *et al.* [1932] found that in skimmed milk cultures with an initial content of 453.7 mg. of insoluble nitrogen (caseinogen, albumin) per 100 ml., the alfalfa organisms effected, during 450 days' incubation, an increase of 29.3 mg. in soluble nitrogen per 100 ml., whilst the corresponding figure for sweet clover bacteria was 19.1 mg. Virtanen [1928] has shown that in water suspensions in the presence of toluene the nodule organisms and crushed nodules cause a slow decomposition of their cell proteins. There is, however, no formation of ammonia in this process, but only of soluble organic nitrogen compounds, with an increase in amino-nitrogen.

The proteoclastic effect of the rhizobia upon different proteins is of particular interest in view of the much-discussed question, how the host plant receives its nitrogen from the root nodules. Virtanen [1928] earlier assumed that the enzymes contained in the bacterial cells caused a breakdown of the cell proteins and that the host plant utilised the amino-acids thus formed in the nodules. It was shown later in this laboratory [Virtanen *et al.*, 1931; 1933] that amino-acids are excreted from the nodules into solid media and that this excretion commences as soon as the nodules are formed, reaching a maximum rate at an early stage of growth (before the plant blooms). These results show that the formation of amino-acids in the nodules is not attributable to the action of the bacterial enzymes upon the cell proteins. Consequently, Virtanen assumed that the excreted amino-acids represent the primary products of nitrogen fixation and that the host plant also utilises these amino-acids. It is interesting to note that, following quite different lines of reasoning, Bond [1933] likewise concludes that the host plant receives from the nodules nitrogenous compounds excreted by the bacteria. According to current views the proteoclastic effect of the rhizobia apparently plays no decisive rôle in the process through which the nodules

supply their host plant with nitrogenous food. Nevertheless, it is interesting to obtain some information regarding the behaviour of these organisms towards different protein compounds.

EXPERIMENTAL.

The proteoclastic effect of the rhizobia was studied with the following substrates: milk, caseinogen, gelatin and autoclaved rhizobia suspensions. Soluble nitrogen was determined in the clear filtrate after the precipitation of proteins by acetic acid in a boiling solution. Amino-nitrogen and ammonia were determined according to Van Slyke, whilst the formaldehyde titration was carried out by Sørensen's method.

Decomposition of milk proteins.

200 ml. of skimmed milk were pipetted into 300 ml. Erlenmeyer flasks, which were then autoclaved at 120° for 20 min. and inoculated, in groups of four flasks, with the following different strains of *Rhizobium*:

<i>Rh. trifolii</i>	VI	(efficient strain)
<i>Rh. trifolii</i>	IV	(weak strain)
<i>Rh. leguminosarum</i>	X	(efficient strain)
<i>Rh. leguminosarum</i>	XVI	(weak strain)

The flasks were plugged with cotton-wool and provided with tight-fitting caps of vegetable parchment to prevent too rapid evaporation. The cultures were incubated at 29°. From each set of four flasks, one culture was analysed after 2, 4 and 6 weeks and again after 6 months (see Table I).

Table I. *Experiment with Rh. trifolii VI.*

	Total N mg./10 ml.	Soluble N % of total N	Amino-N % of soluble N	Formald. titration ml. 0.2 N NaOH/20 ml.	NH ₃ -N % of total N
Initial	36.2	13.3	7.0	4.3	1.0
After 2 weeks	36.3	13.8	8.7	4.0	2.8
After 4 weeks	36.8	14.3	8.7	3.7	2.4
After 6 weeks	37.0	14.3	8.7	3.8	2.2
After 6 months	43.0	22.3	17.0	6.7	1.0

The decomposition of the milk proteins has thus been exceedingly slow. A distinct rise occurred in the values of soluble and amino-nitrogen only after 6 months' incubation. Precisely similar results were obtained with other bacterial strains, so it is not necessary to record these experiments in detail.

The formation of ammonia was very slight. However, in older cultures the content of ammonia must have been higher than is indicated by the figures in Table I, since part of the ammonia escaped by evaporation. This is also evidenced by the fact that the 6 months' culture shows a decreased ammonia content.

Decomposition of caseinogen.

The decomposition of caseinogen was studied both with living cultures and with heavy suspensions in the presence of toluene. The following medium was used in the experiments with living bacteria:

Caseinogen	12 g.	FeCl ₃	Traces
Glucose	6 "	Yeast extract	50 ml.
K ₂ HPO ₄	0.25 "	Tap water	500 "
MgSO ₄ · 7H ₂ O	0.1 "	Agar	2 g.
NaCl	0.1 "	pH	7.4

100 ml. of the semi-solid medium were pipetted into 4 flasks. The cultures were inoculated with *Rh. trifolii* VI and incubated at 29°. The flasks were vigorously shaken daily so that the semi-solid agar medium broke and fresh surfaces were formed for the bacteria to grow on. The growth of the bacteria could thereby be greatly improved.

The initial content of ammonia-nitrogen (per 100 ml. of the medium) was 2.0 mg. The corresponding values after 3 and 6 weeks' incubation were 2.0 and 2.8 mg., respectively. The formation of ammonia was thus quite negligible.

The experiment with a heavy bacterial suspension was likewise made with *Rh. trifolii* VI, grown on 400 gelatin slopes. The gelatin medium was prepared as follows:

Sucrose	5.0 g.	Lupin extract	200 ml.
Asparagine	2.5 „	Tap water	200 „
Gelatin	130.0 „	p _H	7

The gelatin slope cultures were allowed to grow at room temperature for 7 days, after which the bacterial mass was washed off with sterile water and the suspension centrifuged clear. 400 tubes produced about 5 g. of moist bacterial mass, which was suspended in 100 ml. of sterile water: 15 ml. of toluene were added and the flask was thoroughly shaken and allowed to stand for 24 hours. 15 ml. of the well-shaken suspension were then pipetted into 150 ml. of 2% caseinogen solution. This solution contained 10 ml. of toluene and had p_H 6.9. The caseinogen was prepared according to Hammarsten and dissolved in dilute NaOH whereupon the solution was neutralised with acetic acid to p_H 6.9. The rubber-stoppered flasks were incubated at 29°. At various intervals, one culture was taken for analysis (see Table II).

Table II.

	Total N mg./10 ml.	Soluble N % of total N	Amino-N % of soluble N	Formald. titration ml. 0.2 N NaOH/20 ml.	NH ₃ -N % of total N
Initial	28.8	15.8	5.3	1.7	1.0
After 5 days	29.3	21.1	5.4	1.7	1.0
After 24 days	28.7	27.8	8.9	1.9	1.1
After 30 days	30.0	28.5	8.8	1.9	1.1
After 42 days	30.5	29.8	9.8	2.1	0.9
After 60 days	30.7	36.1	11.7	2.3	0.9

The results in Table II show clearly that there has been no production of ammonia. On the other hand, the values of soluble and amino-nitrogen have slowly risen.

Decomposition of gelatin.

The decomposition of gelatin was also studied both with living bacteria and with heavy suspensions in the presence of toluene. In the former case, two different methods were employed. In preliminary experiments, 0.4% of agar was added to the lupin extract-gelatin medium, and 100 ml. of this medium were pipetted into Erlenmeyer flasks. The flasks were plugged with cotton-wool, sterilised and inoculated with *Rh. trifolii* VI. The flasks were then fitted with caps of vegetable parchment and incubated at 29°. On the third day, a distinct

Table III.

	Total N mg./10 ml.	NH ₃ -N mg./50 ml.	NH ₃ -N % of total N	Formald. titra- tion ml. 0.1 N NaOH/20 ml.
Initial	139.7	3.2	0.5	8.9
After 12 days	139.7	10.4	1.5	10.4

growth had appeared on the agar surface, and the flasks were thereafter shaken daily in order to produce fresh surfaces for the bacterial growth (see Table III).

Another experiment on the decomposition of gelatin by living nodule organisms was made with a suspension of *Rh. leguminosarum* X. The bacterial mass was grown for 7 days in a Roux flask, whereupon it was washed off with sterile water and glass beads. The volume of the suspension so obtained was 100 ml.

75 ml. of a medium, containing 30 g. of gelatin and 3 g. of K_2HPO_4 per l. of water (p_H 7.1), were pipetted into nine 150 ml. Erlenmeyer flasks. The flasks were sterilised at 100° on two successive days, whereupon 10 ml. of the well-shaken bacterial suspension were added to each flask. Three of the cultures were used as controls and analysed immediately; three cultures were analysed after 12 days' incubation at 37° and the remaining three after 21 days. Before analysis, the volume of each culture was made up to 100 ml. with distilled water. The mean results of each set of three cultures are given in Table IV.

Table IV.

	Total N mg./10 ml.	NH_3 -N mg./50 ml.	NH_3 -N % of total N	Formald. titra- tion ml. 0.1 N NaOH/20 ml.
Initial	31.3	0.42	0.27	2.5
After 12 days	30.7	0.80	0.52	2.9
After 21 days	31.2	1.13	0.72	3.1

The results show that very small amounts of ammonia are formed from gelatin. The ratio of ammonia-nitrogen to total nitrogen is, even after 21 days, only in the neighbourhood of 1 %. It is not known which particular constituent of gelatin is the source of this insignificant ammonia production.

The decomposition of gelatin in the presence of toluene was studied by pipetting 100 ml. of 5 % gelatin solution (p_H 6.5) into Erlenmeyer flasks, whereupon 10 ml. of toluene were added and the flasks were vigorously shaken and allowed to stand for 24 hours. 10 ml. of the bacterial suspension (see decomposition of caseinogen in the presence of toluene) were then added and the flasks were fitted with rubber stoppers and incubated at 29° (see Table V).

Table V.

	Total N mg./10 ml.	NH_3 -N mg./50 ml.	NH_3 -N % of total N
Initial	61.6	1.6	0.41
After 5 days	61.6	1.7	0.45
After 17 days	61.6	1.6	0.41
After 30 days	61.3	1.6	0.41
After 36 days	61.6	1.7	0.43
After 48 days	61.0	1.6	0.40

There is thus no formation of ammonia in the presence of toluene.

Decomposition of the cell proteins of rhizobia.

The bacterial mass was obtained from 500 gelatin slope tubes in the manner previously described. After centrifuging, the cell mass was suspended in 500 ml. of sterile water, and the suspension was autoclaved at 120° for 20 min. The following medium was then prepared:

Autoclaved suspension	500	ml.	$MgSO_4 \cdot 7H_2O$	0.1 g.
Yeast extract	50	"	$FeCl_2$	Traces
Glucose	5.0	g.	Agar	2.0 g.
K_2HPO_4	0.25	"	pH	7.10
NaCl	0.1	"				

150 ml. of this medium were pipetted into three 150 ml. Erlenmeyer flasks, which were then plugged with cotton-wool and autoclaved at 115° for 20 min. After cooling, the flasks were inoculated with *Rh. trifolii* VI, covered with parchment caps and inoculated at 29°. When a distinct growth was visible the agar surface was broken by shaking the flasks. This was subsequently done every day (see Table VI).

Table VI.

	Total N mg./150 ml.	Soluble N mg./150 ml.	Soluble N % of total N	NH ₃ -N mg./150 ml.
Initial	504*	294	58.3	1.5
After 2 weeks	504	399	79.1	—
After 6 weeks	504	420	83.0	1.5

* Including 7.2 mg. N contained in the yeast extract.

Table VI shows that over 50 % of the nitrogen contained in the bacterial mass was in a soluble form at the start of the experiment. There is a distinct rise in the soluble nitrogen during incubation. On the other hand, no ammonia has been formed.

SUMMARY.

The decomposition of milk proteins, pure caseinogen, gelatin and the cell proteins of the nodule bacteria was studied both with living cultures of different strains of *Rhizobium* and with heavy suspensions in the presence of toluene. The breakdown of the different protein materials was found to be very slow and no differences could be noted between strains of different efficiency. There was a slow increase in the values for soluble and amino-nitrogen. Living rhizobia formed very small amounts of ammonia from milk proteins and from gelatin, whereas no formation of ammonia took place in the presence of toluene. No formation of ammonia could be demonstrated when the cell proteins of the rhizobia were used as substrates for living nodule organisms.

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LXI. THE ISOLATION OF CAROTENE AND STEROLS FROM THE UNSAPONIFIABLE MATTER OF COCKSFOOT.

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FIVE years ago a comprehensive analysis was made of the ether extract of cocksfoot (*Dactylis glomerata*) [Pollard *et al.*, 1931; Smith and Chibnall, 1932]. During the work, β -carotene, xanthophyll and sterols were isolated in good yield and their properties were studied. The results were not considered to be of sufficient importance for publication, since about that time Kuhn and Lederer [1931, 1] showed that "grass" contained only β -carotene, and the spectrographic examination of the sterols had been undertaken by Prof. Heilbron.

A recent investigation of the cocksfoot carotene by Gillam [1935] has however emphasised the purity of the product, and as dried grass is now available commercially in large quantities and as the pure carotene is readily obtained in good yield, it seems worth while to describe the method of isolation employed. Some further investigations on the sterols and a brief description of the xanthophyll are also given.

The principal constituent of most leaf carotenes has been shown to be the β -isomeride by Kuhn *et al.* [1931] and more recently by Miller [1935], Mackinney [1935] and Strain [1935], who have shown that in many grasses it is the only form that can be detected. Among thirteen members of the Gramineae examined by Miller, the bamboo was the only one in which α -carotene could be found. A measurement of the optical rotation of the carotene isolated by the author from cocksfoot was made at the National Institute for Medical Research through the courtesy of the late Dr Dudley. Within the limits of experimental error, the carotene had no rotation, $[\alpha]_{\text{D}} = -10^\circ$. Since α -carotene has $[\alpha]_{\text{D}} = +380^\circ$ and β -carotene has a rotation of zero, the specimen examined appeared to consist only of β -carotene.

The amount of carotene in the grass may be increased very considerably by suitable nitrogen fertilisation, and specimens with a carotene content much higher than in the present case have been examined. The comparative ease with which the carotene has been isolated from the cocksfoot was chiefly due to the previous removal of the wax material. Unless these compounds are separated at the beginning of the preparation, they have a considerable influence on the solubility of the carotene and on its adsorption in chromatographic analysis. This point has been discussed by Strain [1935], who encountered some difficulty in the later stages of the purification.

When these experiments were undertaken, the presence of ergosterol in forage grasses had not been established, and the possibility that this substance might play some part in the production of the antirachitic vitamin of butter fat was considered to be a point of some interest. The chief constituent of the cocksfoot sterol appeared to be sitosterol and no evidence could be found for the

presence of stigmasterol on brominating the mixture by the method of Windaus [1906; 1907]. Spectrographic examination of the sterol by Prof. Heilbron showed that the spectrum of ergosterol was detectable and corresponded to an ergosterol content of approximately 1% of the mixture [Gillam *et al.*, 1933].

Recently, using the method of Windaus and Borgeaud [1928], it has been possible to isolate from the mixture of sterols a substance which has been identified by melting-points and analysis as the pinacol of ergosterol. The amount obtained corresponded to 0.3% of the mixture, but as ergosterol itself gives the pinacol only in 70% yield, the amount isolated from the grass sterol would indicate a minimum content of 0.4% of ergosterol.

Evidence has been brought forward by Waddell [1934] that the antirachitic vitamin from animal sources is not identical with the irradiation product of ergosterol, and that the pro-vitamin present in specimens of cholesterol gives on irradiation a product of greater antirachitic power than that derived from ergosterol. Furthermore, Windaus [1935] has shown conclusively that some sterols with the same system of conjugated double bonds as ergosterol, but differing in the constitution of the side chain, have properties very similar to those of ergosterol and give rise to irradiation products possessing antirachitic activity.

The identities of compounds of this nature cannot be determined with certainty by a comparison of melting-points alone, and the melting-points of mixtures of such compounds do not necessarily show a depression. Again, the absorption spectrum of 22:23-dihydroergosterol has been found by Windaus [1935] to be identical with that of ergosterol itself.

The possibility still exists therefore that the substance in the cocksfoot sterol may be not ergosterol itself, but some very closely related sterol, differing perhaps only in the constitution of the side-chain. Since however the sterol from cocksfoot gives a pinacol and its acetate which both correspond exactly in melting-point with those derived from ergosterol, and since the absorption spectra are the same, the conclusion that the substance is ergosterol appears to be justified.

EXPERIMENTAL.

Preparation of the light petroleum extract of cocksfoot. Fresh cocksfoot was dried in the experimental grass drying plant of the Imperial (Chemical Industries at Jealott's Hill. Under these conditions, the grass was dried in about 8 min. without scorching, and no breakdown of the carotene occurred. 32.7 kg. of the dried grass were extracted with light petroleum by British Drug Houses and gave a dark yellow-green extract containing 797 g. of solids, as found by the evaporation of an aliquot portion. This was equivalent to 2.44% of the dry weight of the grass.

Separation of wax and phosphatide. The extract was concentrated to a thick syrup and poured into 7 l. of boiling acetone to precipitate the wax and phosphatide. After cooling to 7° the solution was filtered at the pump and the precipitate taken up in 1 l. of warm ether and again precipitated with 2 vols. of acetone. The acetone solutions containing the glycerides, unsaponifiable matter and pigments were combined and evaporated under reduced pressure and the residue was dissolved in light petroleum.

Colorimetric estimation of the pigments in the solution showed the presence of 10.7 g. of carotene equivalent to 0.033% of the dry grass and 7 g. of xanthophyll equivalent to 0.021% of the dry grass, the comparatively small amount of xanthophyll being due to its low solubility in the light petroleum used for the extraction.

Separation of carotene. The light petroleum solution was diluted with the same solvent to a total volume of 7 l. and the solution divided into three equal parts. Each portion was shaken with successive quantities of 92 % methanol which had previously been saturated with light petroleum, using in all 12 l. of methanol. A carotene solution in petroleum and a xanthophyll solution in methanol were thus obtained, and at the same time the carotene solution had been freed from a considerable part of the chlorophyll and of the general unsaponifiable material, which had passed over into the methanol. The greenish brown petroleum solution was divided into three equal parts. The first was shaken mechanically for half an hour with 200 ml. of a saturated solution of potassium hydroxide in methanol, the light petroleum solution decanted and shaken with another 200 ml. of alkali. The second alkaline solution was used for the first extraction of the next portion of the light petroleum solution and the process continued, so that eventually each portion of the light petroleum had been shaken with three successive amounts of alkali. The petroleum solutions were then combined and concentrated under reduced pressure to a thick syrup which was taken up in ether. The ethereal solution was then washed with water and dried over sodium sulphate.

From this point onwards all manipulations and crystallisations were carried out as far as possible in an atmosphere of nitrogen or carbon dioxide, and the solutions were kept in the dark. The deep red ethereal solution was concentrated to a syrup under reduced pressure and diluted with absolute alcohol to a total volume of 300 ml. In a short time carotene began to separate in small glistening crystals. These were collected at the pump and further crops were obtained by the addition of absolute alcohol to the mother-liquors. The last fractions of carotene were contaminated by small quantities of wax and sterol, which were removed by washing with warm alcohol and ether. The mother-liquors were concentrated and again treated with alcohol, when further small quantities of carotene were obtained. The crude carotene, which still contained a small amount of colourless impurities, amounted to 7 g. and colorimetric estimation showed that the mother-liquors still contained 2.7 g. of carotene in solution.

The carotene was dissolved in 1400 ml. of warm benzene and a few ml. of methanol added. On cooling, 4 g. carotene crystallised out in the form of large red crystals with a green lustre, which were collected by filtration and kept in an atmosphere of nitrogen in the dark.

The carotene had m.p. 182° (corr.), and I have to thank Mr Gillam for examining the material spectrographically. He found that the intensity of its absorption was about 10 % higher than that of the best specimen which he had examined previously and that it gave a value of $E_{1\text{ cm}}^{1\%}$ (463 $m\mu$) = 2200 in chloroform solution. In light petroleum (B.P. 60–80°) the corresponding value was 2500 [Gillam, 1935]. This intensity of absorption is similar to or slightly less than that of Kuhn's β -carotene [Smakula, 1934; Kuhn and Lederer, 1931, 2].

Recently Ferguson [1935] has used a specimen of the same carotene to construct curves for use in the colorimetric estimation of carotene. The colour was compared against the yellow units of the Lovibond tintometer (B.D.H. Pattern) and against the usual potassium dichromate solution.

All the mother-liquors from the carotene solutions were combined, evaporated and found to contain 66 g. of a red gummy material. When this was stirred with a small quantity of 95 % alcohol and kept in the refrigerator, small quantities of sterol separated. After removal of the sterol, the solution was again concentrated and taken up in a small quantity of light petroleum. Further amounts of

crystalline sterol were obtained, giving in all 7 g. of crude material. The liquid residue still contained 5.8 g. of sterol as estimated by digitonin.

Xanthophyll. The methanol solutions containing the xanthophyll were combined and concentrated *in vacuo* to about 8 l. This solution was saponified in portions of 2 l. by boiling under a reflux condenser with 100 g. KOH for 1.5 hours. The methanol was distilled off *in vacuo* and the combined residues (1500 ml.) diluted with water and extracted with ether. The ethereal solution (6 l.) was washed with water, dried over sodium sulphate and concentrated to 300 ml. On pouring this solution into 2 l. of light petroleum, 2 g. of xanthophyll separated and were collected at the pump. The mother-liquor was concentrated to a syrup (44 g.) and on treatment with light petroleum (100 ml.) and absolute alcohol (300 ml.) gave 10 g. of crystalline sterol. The residual solution was partitioned between light petroleum and 90 % methanol, and from the methanol solution, which contained the greater part of the original red colour, small quantities of xanthophyll were obtained on concentration and treatment with light petroleum as before. The mother-liquors were estimated to contain 2 g. of xanthophyll and 2.8 g. of sterol.

The xanthophyll (2.5 g.) crystallised from benzene in dark red crystals, M.P. 174° . On further recrystallisation from chloroform-methanol it melted at 182° . The material appears to be the usual mixture of xanthophylls and to consist largely of lutein. Although the crystalline fractions gave no colour in ethereal solution with 25 % HCl, the mother-liquors gave the characteristic blue colour given by violaxanthin, which has been shown to be present in the great majority of leaf xanthophylls by Kuhn *et al.* [1931]. Analysis of the crystals, M.P. 182° (uncorr.), corresponded with that of dihydroxyxanthophyll. (Found: C, 83.9; H, 10.0 %. $C_{40}H_{56}O_2$ requires C, 84.4; H, 9.93 %. $C_{40}H_{56}O_4$ requires C, 82.08; H, 9.40 %.)

Sterols. The several fractions of crude sterol were combined (19.5 g.), dissolved in 95 % alcohol and filtered from a small quantity of carotene. The sterol was recovered from the solution and recrystallised from acetone, methanol and light petroleum. Finally 12.85 g. of sterol were obtained, M.P. $138-139^{\circ}$ (uncorr.) and $[\alpha]_D = -36^{\circ}$.

Isolation of ergopinacol. The sterol (10 g.) was dissolved in a mixture of absolute alcohol (350 ml.) and benzene (50 ml.) and cosin (1 g.) added. The solution was boiled for 5 min. to expel dissolved oxygen, stoppered and after cooling was exposed overnight to the light from a 150-watt electric bulb. On cooling to room temperature the ergopinacol separated in characteristic films of small needle-shaped crystals, which were collected and recrystallised from pyridine-alcohol. The ergopinacol was obtained as fine needles and weighed 30 mg. When melted side by side with an authentic specimen, both substances melted at 201° . The acetate, first obtained by H. H. Inhoffen in Göttingen (unpublished), was prepared by heating the pinacol with a few ml. of pyridine and acetic anhydride for half an hour on a boiling water-bath. On adding a trace of water and allowing the solution to cool, the acetate crystallised and was collected. After recrystallisation from a mixture of chloroform and alcohol the substance formed colourless needles, M.P. 204° . A specimen prepared from the authentic ergopinacol melted at 205° , while a mixture of the two melted at 204° . (Found: C, 82.3; H, 10.4 %. $C_{80}H_{90}O_4$ requires C, 82.0; H, 10.2 %.) As the melting-points of the pinacols and derivatives are dependent on the rate of heating, all specimens for comparison were melted side by side; the recorded values are consequently uncorrected.

SUMMARY.

The light petroleum extract of a common forage grass, cocksfoot, has been investigated.

From the unsaponifiable fraction carotene, xanthophyll and sterols have been isolated.

The carotene appeared to consist entirely of the β -isomeride and has been isolated in a state of purity. The xanthophyll, which was a mixture, has not been further investigated.

From the sterol fraction, which consisted chiefly of sitosterol, ergosterol has been isolated as the pinacol in an amount corresponding to 0.4 % of the total sterol. The presence of approximately 1 % of ergosterol had previously been determined spectrographically.

The problem of the identification of such substances is discussed.

The author has great pleasure in thanking Prof. A. C. Chibnall for his interest and advice during the course of the work, and Dr H. H. Inhoffen for his assistance in the isolation and identification of the ergopinacol and for many helpful discussions.

He also wishes to thank the Imperial Chemical Industries for a grant which made the investigation possible.

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LXII. FAT METABOLISM IN FISHES.

IX. THE FATS OF SOME AQUATIC PLANTS.

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It has been shown [Lovern, 1935, 1] that fats from several plankton crustacea exhibit the same type differences between marine and freshwater species as are shown by fish fats, namely more C_{16} and C_{18} and less C_{20} and C_{22} unsaturated acids in the freshwater species. Indeed, so far as the animal chain is concerned, from the vegetable-feeding plankton upwards, the evidence is in harmony with the idea that ingested fat may be deposited largely unchanged in most cases. It is of interest to know whether the plankton crustacea may also deposit ingested fat unchanged, or whether they are forced to modify it. There is also the question of whether the type difference between marine and freshwater species is confined to the animal kingdom or extends to aquatic plant life.

In the present paper the results of the detailed analyses of the fats from several species of algae, both marine and freshwater, are described. In addition the fats from a higher aquatic plant and from a marine diatom have been examined. So far as the author is aware the only data so far available regarding such fats are qualitative or at most semiquantitative. The content of fatty matter in marine algae has been shown to depend mainly on the depth of immersion [Russell-Wells, 1932; Haas and Hill, 1933]. Tsujimoto [1925] showed by bromination that algal fats contained small quantities of highly unsaturated acids. Takahashi *et al.* [1933] identified *n*-hexanoic, *n*-octanoic, *n*-decanoic, linoleic, oleic, and myristic acids in certain algal fats. Collin *et al.* [1934] found that fatty acids from mixed phytoplankton gave only a small yield of ether-insoluble bromides.

EXPERIMENTAL.

Large quantities of the marine algae *Fucus vesiculosus*, *Laminaria digitata*, *Rhodomenia palmata* and a small quantity of mixed green species were collected near Aberdeen. Green marine algae are not easy to obtain in quantity, and the mixture was needed to obtain sufficient fat for analysis. Supplies of the freshwater algae *Nitella opaca* and *Oedogonium* sp. were supplied through the kindness of Dr R. L. A. Beauchamp of the Freshwater Biological Association of the British Empire. With the assistance of the Great Yarmouth Port and Haven Commissioners a large supply of the freshwater alga *Cladophora sauteri* was obtained from Hickling Broad. The higher aquatic plant *Anacharis alsinastrum* (Canadian pondweed) was obtained in quantity from the Loch of Pitfour, Aberdeenshire, by the kind permission of Mr F. Martin of Mintlaw, Aberdeenshire. The marine diatom *Nitzschia closterium* was grown in pure culture until about 13 g. of fat were available.

The algae and the pondweed (entire plant) were all extracted with ether after air-drying and powdering. The *F. vesiculosus* was extracted at Manchester by Prof. I. M. Heilbron in the course of his work on algal constituents and the fatty acids were forwarded to Aberdeen. In this case two extractions were made,

first with alcohol and then with ether, the fatty acids from each of these extracts being examined separately. All the other dried materials were extracted with ether only and in every case except the pondweed the extract was freed from phosphatides with acetone. The proportions of phosphatides were always very low and the pondweed results can safely be taken as those of the glyceride fraction only. The diatoms were filtered out and the whole mass, including paper, was extracted first with alcohol and then, after mincing, with ether. The extracts were combined and this fat was also freed from phosphatides.

The samples were examined by substantially the same process as that used hitherto for fish fats, but the presence of large quantities of chlorophyll (which cannot be altogether removed during saponification, removal of unsaponifiable matter and acid recovery) necessitated purification of the fatty acids with light petroleum. Small quantities of chlorophyllins which remained accumulated in the residues during distillation and were removed by again extracting the residues with light petroleum. The possible presence of lower fatty acids such as *n*-decanoic acid also involved extra precautions. It was further found that the pondweed fat was contaminated with appreciable quantities of wax acids (both saturated and unsaturated) similar to those reported in certain grass fats [Lovern, 1935, 2]. All but one of the algae appeared to be free from waxes, but *C. sauteri* contained traces. The fat contents of the various materials were not accurately determined, but it was evident that the depth effect previously mentioned was correct.

The results of the fatty acid analyses are given in Table I. The wax acids have been eliminated from the two cases where they were present, and in every case the composition recorded is that of the glyceride fatty acids. These plant fats are

Table I. *Composition of mixed fatty acids (wts. %).*

Class	Species	Saturated				Unsaturated				
		C ₁₂	C ₁₄	C ₁₆	C ₁₈	C ₁₈	C ₁₈	C ₂₀	C ₂₂	C ₂₄
Chlorophyceae	<i>N. opaca</i> *	—	6	18	3	3 (-2.0H)	31 (-2.5H)	23 (-1.5H)	13 (-5.8H)	—
	<i>Ordogonium</i> sp.*	—	2	20	1	—	32 (-3.1H)	35 (-1.6H)	9 (-2H)	1 (-2H)
	<i>C. sauteri</i> *	Trace	12	10	2	Trace	19 (-1.7H)	49 (-3.8H)	8 (-7.1H)	—
	Mixed†	—	4	10	2	3 (-2.0H)	30 (-3.4H)	30 (-5.1H)	8 (-6.5H)	4 (-2H)
Phaeophyceae	<i>F. vesiculosus</i> (1)†	Trace	8	9	1	Trace	6 (-2.0H)	57 (-3.2H)	16 (-7.3H)	3 (-2H)
	<i>F. vesiculosus</i> (2)†	Trace	9	7	2	1	5 (-2.0H)	63 (-3.0H)	13 (-7.3H)	—
	<i>L. digitata</i> †	—	6	14	1	2	11 (-2.0H)	42 (-4.2H)	24 (-8.1H)	—
Rhodophyceae	<i>R. palmata</i> †	1	4	19	1	Trace	6 (-2.0H)	20 (-4.5H)	36 (-9.2H)	13 (-2H)
Higher plant	<i>A. alsinastrium</i> *	1	1	15	5	2	25 (-3.0H)	39 (-4.9H)	12 (-6.0H)	—
Diatom	<i>N. closterium</i> †	—	8	17	2	1	36 (-3.4H)	20 (-5.3H)	16 (-7.0H)	—

* Freshwater species.

† Marine species.

more difficult to deal with and the experimental error is likely to be higher than with fish fats. In addition several fats had to be examined on a small scale and so the results are only expressed to the nearest unit. The degrees of average unsaturation are given in terms of lack of hydrogen.

The nature of the acids of less than 14 carbon atoms was not determined in most cases, but in *R. palmata* both *n*-decanoic and *n*-octanoic acids and also smaller traces of an unsaturated acid of less than 14 carbon atoms were present. *F. vesiculosus* (1) and (2) are from the alcohol and ether extracts respectively.

DISCUSSION.

From Table I it can be seen that for these plant fats the distinction between freshwater and marine species does not hold. In the case of the algae the properties seem to follow the colour grouping. Of the green algae three were freshwater species and one marine. This mixed marine specimen was really from brackish water rather than pure seawater, since green algae grow more prolifically near the mouths of rivers than in pure seawater, and this sample was collected in such brackish areas. However, if salinity has any effect on the fat it should have shown in this sample and it is obvious that actually this fat is of the same type as those of the other Chlorophyceae.

The saturated acid percentages show great irregularities throughout, but for the unsaturated acids some interesting correlations occur. For the green algae (Chlorophyceae) the predominating unsaturated acids are those of 16 and 18 carbon atoms, with little C_{20} and little or no C_{22} acids. The degrees of average unsaturation of the C_{16} and C_{18} acids are unusually high. In the brown algae (Phaeophyceae), C_{16} unsaturated acids have not the same importance, C_{18} acids are outstanding and C_{20} acids are present in somewhat greater proportions than for most green algae. The C_{16} acid is monoethylenic only, and the C_{18} acids are not on the whole of an unusually high degree of unsaturation. For the one red alga (Rhodophyceae) examined C_{20} acids are the major constituent and appreciable quantities of C_{22} acids are present. C_{16} unsaturated acids, whilst not present in large amounts, are again of a relatively high degree of average unsaturation.

It is perhaps not unexpected that plant fats will fall into classes according to botanical similarities rather than according to habitat, since this is so markedly the case for seed fats. It is interesting to note that Heilbron *et al.* [1935] found that the components of the unsaponifiable matter from algae were decided by whether the alga was a green, red or brown species.

Turning to the higher plant *Anacharis alsinastrum* we find a fatty acid mixture very similar to that of green algae. The whole question of the composition of leaf, stalk *etc.* fats requires examination, especially in view of the author's results on two land grass fats [Lovern, 1935, 2] where a somewhat similar type of fat was found.

The marine diatom *Nitzschia closterium* has a fatty acid mixture also closely resembling that of a green alga, although this diatom is brown in colour.

Taking these fats as a whole it may be said that the fats of all the green algae, the pondweed and the diatom are of a type very similar in many respects to freshwater animal fats. The brown algal fats are really of a class by themselves, but more like a freshwater than a marine animal fat. The red algal fat is the only one approximating in composition to a marine animal fat.

It has already been mentioned that throughout the animal chain to the copepods and other plankton crustacea, the same general types of fat are found for freshwater and marine organisms respectively. These plankton crustacea do not live on the large attached algae, but on diatoms and the smaller floating algae. If *N. closterium* is typical of most marine diatoms it is evident that such copepods as *Calanus finmarchicus*, for example, must considerably modify ingested fat. *C. finmarchicus* contains high percentages of C_{20} and C_{22} acids [Lovern,

1935, 1]. The work of Collin *et al.* [1934] suggests that *N. closterium* is typical of other marine diatoms since a mixture of forms gave fatty acids of which only 2.1% gave bromides insoluble in ether. C_{20} and C_{22} highly unsaturated acids could not have been present in large amounts, especially as some of the C_{18} acids (if similar to those of *N. closterium*) would have contributed to the ether-insoluble bromides. If the small floating algae resemble the larger attached forms, the marine zooplankton cannot obtain much of its C_{22} acids from them either.

Turning to the freshwater organisms it is regrettable that attempts made to grow a freshwater diatom in pure culture were unsuccessful, and so no analysis comparable with that of *N. closterium* fat is available. However, it has been shown that for plants the distinction between freshwater and marine forms does not hold, but that botanical classification is closely correlated with type of fat. It is not unreasonable therefore to take *N. closterium* fat as typical of most diatom fats, whether marine or freshwater. If this is so, all the freshwater plants—diatoms, algae and higher plants—have fats closely similar to freshwater animal fats and in this case it is possible that the plankton crustacea deposit ingested fat largely unchanged in type. In freshwater some of the larger plant forms also contribute to the animal food, *via* the medium of herbivorous fish, molluscs *etc.*, and here also there is no reason for ingested fat to be modified to any great extent.

SUMMARY.

The proportions of the various fatty acids in a number of algal fats have been examined. The fats fall into groups agreeing with their botanical relationships, and no difference between salt- and fresh-water forms as such could be detected. A marine diatom and a higher aquatic plant both had fats similar to those of green algae.

The results suggest that whilst freshwater plankton crustacea may deposit ingested fat largely unchanged in type, the corresponding marine organisms are forced to modify it considerably.

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LXIII. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES.

III. THE CATION CONTENT OF ERYTHROCYTES OF RABBIT'S BLOOD IN HYPER- AND HYPO-TONIC SERA.

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IN earlier papers of this series [Davson, 1934; Davson and Danielli, 1936] attention has been drawn to the work of Ponder and Saslow [1930; 1931] on the volumes of rabbit's erythrocytes. These authors observed that in hypotonic solutions the erythrocytes gained much less water than was expected and in hypertonic solutions lost far less; in fact an increase in volume of the erythrocytes was generally observed instead of the expected contraction. These anomalous results have been interpreted by Ponder and Saslow as being due to the penetration or loss of cations and have been considered as evidence that the erythrocyte may become completely permeable to cations. Although the author [1934] has found a certain penetration of K^+ and Na^+ in hypertonic solutions with the ox erythrocyte, none of the order of magnitude inferred by Ponder and Saslow was ever found. It was decided therefore to repeat some of the experiments of Ponder and Saslow determining both K^+ and Na^+ and volume changes. It was found that although the changes in volume are not so great as those to be expected on the basis of complete cationic impermeability, the changes are in the right direction and the anomalies are by no means so great as those described by Ponder and Saslow.

EXPERIMENTAL.

Hypotonic experiments. Defibrinated rabbit's blood taken from the carotid under ether anaesthesia was measured into centrifuge-tubes; the erythrocytes were centrifuged, water was added and the erythrocytes were mixed with the diluted serum. After one hour the suspensions were centrifuged again and 1 ml. of erythrocytes was weighed out for the determinations of cations and 2 ml. for the determination of the water content. A haematocrit determination on the original blood gave the proportion of serum to whole blood and thus enabled the calculation of the degree of hypotonicity. For the K^+ determination it was found impossible to haemolyse the erythrocytes completely by adding distilled water, so that in the ashed trichloroacetic acid filtrates low values for K^+ were obtained; the erythrocytes were therefore ashed whole.

Hypertonic experiments. No preliminary centrifuging was necessary; 2-5 ml. of a 4% solution of KCl were added to the blood. The rest of the procedure was as above.

RESULTS.

In Table I the results of representative experiments are shown. The relative volumes in column 4 are calculated from the changes in water content. In column 5 the cation contents are recorded as determined by comparing the

Table I.

Exp.	Tonicity compared with normal of rabbit serum	% H ₂ O	Volume		Cations (millimols.)	
			Found	Expected	Found	Expected
1	1	66.7	100	100	111	111
	1.76	62.1	88	76	112	143
	2.56	58.6	80	63	111	173
2	1	62.0	100	100	110	110
	0.84	64.3	109	110	110	103
3	1	68.7	100	100	111	111
	0.68	73.5	124	121	110	113

relative volumes of the test and control corpuscles: thus in Exp. 1 the cation content is 111 millimols. per 100 g. in the control, and 112 and 111 millimols. per 88 g. and 80 g. respectively in the test solutions. In this way the cation content is referred to a fixed number of corpuscles so that any changes in its value must be caused by penetration or loss of cations. In the last column is shown the cation content expected on the basis that the anomalies in the volumes are explicable by the penetration or loss of cations. It is evident that in hypertonic solutions the volume changes differ from those expected whereas in hypotonic solutions the changes are approximately in accordance with expectation. The close agreement between the cation contents in control and test experiments is illusory as considerable errors must be introduced by failure to pack the corpuscles thoroughly. Thus in an experiment (not shown) in which the hypertonicity was produced by addition of NaCl instead of KCl an apparent increase of nearly 6% in the K⁺ content of the erythrocytes occurred; as no extra K⁺ had been added to the serum this apparent increase must have been due to underestimation of the shrinkage; however, even when the shrinkage was corrected the discrepancy between the experimental and theoretical values was of the same order as that found in the experiments shown.

DISCUSSION.

Two facts emerge from the results described here. First that the abnormalities observed by the author are not so large as those described by Ponder and Saslow. Secondly that the abnormalities observed are not to be correlated with the penetration of K⁺ or Na⁺. In discussing a possible free penetration of both cations and anions into the erythrocyte the following point should not be neglected. The cells suspended in isotonic KCl solution may be represented thus:

Hb ⁻		K ⁺
Cl ⁻		Cl ⁻
K ⁺		
Cells (1)		Serum (2)

If now the cells are freely permeable to both K⁺ and Cl⁻ the equation representing the Donnan equilibrium is

$$[K^+]_1 [Cl^-]_1 = [K^+]_2 [Cl^-]_2 \\ = [Cl^-]_2^2.$$

Or since $[K^+]_1 > [Cl^-]_1$ $[K^+]_1 + [Cl^-]_1 > 2 [Cl^-]_2$.

Hence there must always be a difference in osmotic pressure between cells and serum; to compensate this water will penetrate into the cells, but in virtue

of the assumed diffusibility of both ions more KCl will penetrate and so the process will go on until the cells burst. If on the other hand only one ion is diffusible no such relation is obtainable, in fact it may be shown that osmotic equality must exist between cells and serum. Thus it is evident that the erythrocyte maintains its integrity precisely in virtue of the indiffusibility of its cations. Hence Ponder and Saslow's contention that the erythrocytes may become completely permeable to cations in hypertonic solutions is untenable without further assumptions.

Takei [1921] states that the erythrocytes of the rabbit behave like perfect osmometers in hypertonic solutions up to 4 % NaCl and then behave anomalously in that they swell and haemolyse to a certain extent. Ege [1923] on the other hand disputes these conclusions both on theoretical and experimental grounds. Thus the state of affairs is still very confused, but it seems from the results described here that the anomalies in hypertonic solutions cannot be ascribed to the penetration of K^+ or Na^+ .

I am indebted to Prof. Drummond for his interest and advice in this work.

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LXIV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLVIII. PENICILLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM PUBERULUM* BAINIER AND *P. CYCLOPIUM* WESTLING.

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DURING an investigation of the possible connection between the incidence of pellagra and mould deterioration of maize Black and Alsberg [1910] and Alsberg and Black [1913] isolated a hitherto undescribed mould metabolic product which they named penicillic acid, $C_8H_{10}O_4$. The constitution of this substance, which was separated from cultures of *Penicillium puberulum* Bainier, isolated from mouldy maize and grown on Raulin's medium, and which was somewhat toxic to mice, was not investigated. Through the courtesy of Dr Charles Thom, we obtained Alsberg and Black's culture of *P. puberulum* about seven years ago and were able to repeat their isolation of penicillic acid, though in diminished yield. It was found later [Birkinshaw and Raistrick, 1932] that the yield of penicillic acid had become very small indeed even on Raulin's medium, so that work on its molecular constitution had to be abandoned. Fortunately we have recently discovered during an investigation of the metabolic products of *Penicillium cyclopium* Westling [Oxford and Raistrick, 1935] that this organism produces relatively large amounts of penicillic acid, and hence we have been enabled to complete the investigation. It is worthy of note that *P. puberulum* and *P. cyclopium* are not closely related, morphologically.

The significant properties of penicillic acid are as follows.

It crystallises from light petroleum as $C_8H_{10}O_4$, and from water in the hydrated form $C_8H_{12}O_5$ and is optically inactive.

The anhydrous acid yields only one active hydrogen atom (Zerewitinoff), it contains one acetylatable hydroxyl group and one methoxyl group (Zeisel).

The presence of at least one double bond is indicated by formation of a dibromo-derivative $C_8H_{10}O_4Br_2$ and of a dihydro-derivative separating from non-aqueous solvents as $C_8H_{12}O_4$ and from water as $C_8H_{14}O_5$ (cf. the analogous behaviour of penicillic acid). A second molecule of hydrogen is absorbed more slowly, but no crystalline tetrahydro-derivative could be isolated.

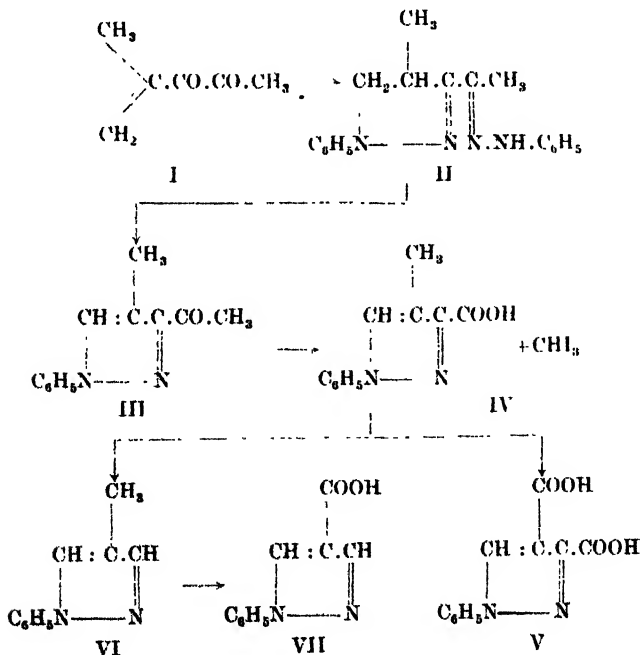
Penicillic acid probably contains a carboxyl or lactone group since it titrates as a monobasic acid and CO_2 is lost with comparative ease. Methylation of dihydropenicillic acid (diazomethane) gives an oily neutral methyl ester, $C_9H_{14}O_4$, whilst similar treatment of either anhydrous or hydrated penicillic acid leads to brisk evolution of nitrogen with formation of a neutral compound $C_{10}H_{14}O_4N_2$. The constitution of this compound is dealt with later (p. 397) but it is evident that during the reaction penicillic acid has combined with a molecule of diazomethane at a double bond in addition to gaining a methoxyl group by esterification of the acidic group.

Ozonisation of penicillic acid gives, in addition to other products to be discussed later, formaldehyde, indicating the presence of a terminal methylene grouping. Dihydropenicillic acid gives no formaldehyde with ozone indicating that the linkage of the terminal methylene group is reduced on hydrogenation.

Neither penicillic nor dihydropenicillic acid gives direct ketonic reactions in aqueous solution. On the other hand hydroxylamine titration of penicillic acid shows a rapid uptake of 1 mol. and a much slower uptake of a second mol. of hydroxylamine, whereas dihydropenicillic acid absorbs no hydroxylamine under the same conditions.

Considerable light was thrown on the constitution of penicillic acid by a study of the yellow crystalline derivative obtained by the action of phenylhydrazine on penicillic acid and originally described by Alsberg and Black. This product has the empirical formula $C_{18}H_{20}N_4$ (II) and gives a strong Knorr's reaction for pyrazolines (an intense purple colour with sulphuric acid and potassium dichromate). It is evidently a compound of 2 mols. of phenylhydrazine with a breakdown product $C_6H_8O_2$ (I), which is derived from penicillic acid $C_8H_{10}O_4$ by loss of CH_2 (i.e. a methoxyl group) and CO_2 (i.e. the carboxyl group). The empirical formula $C_{18}H_{20}N_4$ (II) agrees with the initial production of a bisphenylhydrazone of a diketone (or ketone-aldehyde or dialdehyde) containing one double bond, with subsequent rearrangement involving one phenylhydrazone grouping and leading to the formation of a pyrazoline ring to give compound (II). On oxidation of (II) with lead dioxide in acetic acid and subsequent treatment with sulphuric acid in the cold there is formed a colourless pyrazole $C_{12}H_{12}ON_2$ (III). Hydrolysis has thus eliminated 1 mol. of phenylhydrazine which must therefore have been combined with an aldehyde or ketone group (now liberated) in the form of a phenylhydrazone.

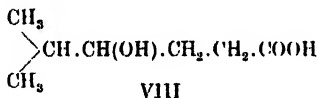
The pyrazole (III) on oxidation with alkaline iodine yields iodoform and a pyrazolemonocarboxylic acid $C_{11}H_{10}O_2N_2$ (IV), which is further oxidised by



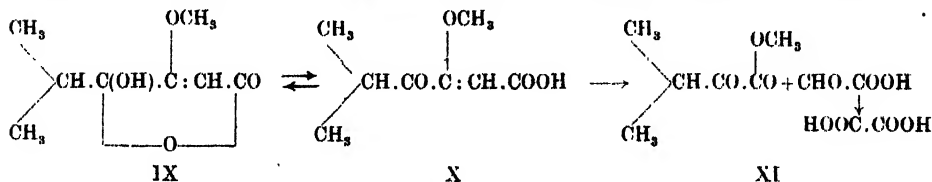
potassium permanganate to a dicarboxylic acid, $C_{11}H_8O_4N_2$. This compound was shown by synthesis to be 1-phenylpyrazole-3:4-dicarboxylic acid (V). The substituents in the original pyrazole (III) must therefore be $\cdot CH_3$ and $\cdot CO \cdot CH_3$, and hence this pyrazole must be either 3-acetyl-4-methyl- or 4-acetyl-3-methyl-1-phenylpyrazole. The position to be allotted to each substituent was settled by decarboxylation of the pyrazolemonocarboxylic acid (IV). The resulting 1-phenylmethylpyrazole (VI) on oxidation with potassium permanganate gave a 1-phenylpyrazolecarboxylic acid (VII), M.P. 219–221°. This M.P. is assigned in the literature (*cf.* Wislicenus and Bindemann, [1901]) to the 1-phenylpyrazole-4-carboxylic acid, the 3- and 5-carboxylic acids having M.P. 146° and 183° respectively. Thus the original methyl group in (III) is in the 4-position, and hence (III) must be 3-acetyl-1-phenyl-4-methylpyrazole. The breakdown product $C_6H_8O_2$ (I) from which it arises is therefore $\beta\gamma$ -diketo- δ -methylene-pentane. The reactions are formulated above.

It now remains to decide the positions of attachment in the $\beta\gamma$ -diketo- δ -methylene-pentane of the methoxyl and carboxyl groups which are eliminated from penicillic acid by treatment with phenylhydrazine.

The carboxyl group would be expected to occupy a position β to one of the keto-groups in order to explain its ready removal. This was confirmed and its position was fixed by a study of the reduction products obtained by the action of hydriodic acid on dihydropenicillic acid. A lactone was obtained in small yield giving a crystalline phenylhydrazone, which was shown by synthesis to be the phenylhydrazone of γ -hydroxy- δ -methylhexanoic acid (VIII).

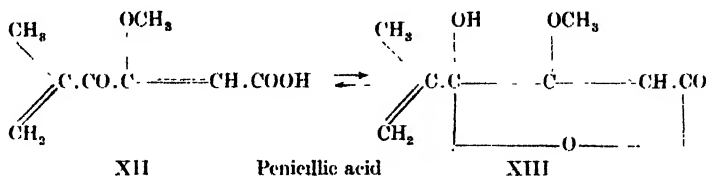


The position of the methoxyl group in penicillic acid was decided from a study of the oxidation of dihydropenicillic acid (IX and X) with potassium permanganate and with ozone to methyl dimethylpyruvate (XI) which was isolated as its 2:4-dinitrophenylhydrazone. Permanganate yields oxalic acid in addition. The methoxyl group is therefore attached to the β -carbon atom in the $\beta\gamma$ -diketo- δ -methylene-pentane structure (I). We must therefore suppose that the β -keto-group is methylated in dihydropenicillic acid (IX and X) in its enol form which would give a double linking between the α - and β -carbon atoms. Hence dihydropenicillic acid (keto-form X) contains both a potential β -keto- and a true γ -keto-group, so that any reaction which would demethylate penicillic acid would thereby render it susceptible to decarboxylation. The other product of the oxidation should be glyoxylic acid; assuming that this is further oxidised to oxalic acid by the excess of oxidising agent present, we may formulate the reaction



The course of the oxidation is good evidence for a double linking between the α - and β -carbon atoms. Further, since oxidation of penicillic acid with ozone yields formaldehyde the parent substance must contain a terminal $=CH_2$ group.

constitution of anhydrous penicillic acid may therefore be written, in its simplest form, as a substituted γ -keto-acid of structure (XII). In this form penicillic acid is thus γ -keto- β -methoxy- δ -methylene- Δ^5 -hexenoic acid. In order to explain its many properties we must also postulate that it can exist as a γ -hydroxylactone of structure (XIII).



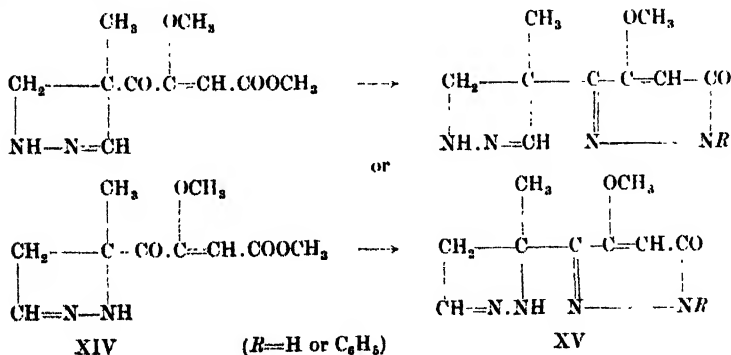
Chemical evidence for the existence of the keto-form (XII) with a free carboxyl group is afforded by the ready esterification of anhydrous penicillic acid with diazomethane and by the ready reaction of penicillic acid with one molecule of free hydroxylamine in the cold in aqueous solution.

Evidence for the lactone form (XIII) is found in the production of a neutral acetyl derivative by the action of acetic anhydride on penicillic acid.

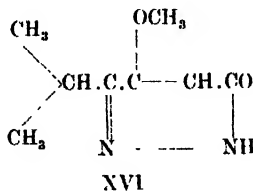
Analogies for the existence of γ -keto-acids in tautomeric forms are to be found in the behaviour, *e.g.*, of laevulic acid, maleic aldehydo-acid and the trialkyl-succinaldehydo-acids (for discussion see Houben-Weyl [1930]). We therefore feel justified in postulating that penicillic acid is capable of existence in two tautomeric forms, (XII) and (XIII).

In the case of dihydropenicillic acid we again have evidence for both carboxylic and lactone forms (IX and X). This compound reacts with diazomethane to give a methyl ester but in aqueous solution is an extremely weak acid and does not react with hydroxylamine in the cold. Dihydropenicillic acid, unlike penicillic acid, apparently exists almost entirely in the lactone form in aqueous solution.

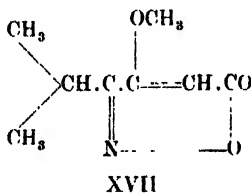
The action of diazomethane on penicillic acid, giving rise to the compound $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_2$ referred to on p. 394, is readily explained from formulae (XII) and (XIII). A pyrazoline ring has been formed by the addition of CH_2N_2 at the double bond in the δ -methylene group to give (XIV). When (XIV) is treated with hydrazine or phenylhydrazine, crystalline products are obtained having the empirical formulae $\text{C}_9\text{H}_{12}\text{O}_2\text{N}_4$ and $\text{C}_{15}\text{H}_{16}\text{O}_2\text{N}_4$ (XV) respectively, which correspond with the addition of the constituents and loss of methyl alcohol. We therefore postulate that an orthodiazine ring is formed, the product being a pyridazone. This reaction is characteristic of γ -ketocarboxylic acids and is the analogue of pyrazolone formation by β -keto-esters.



The position of attachment of diazomethane to penicillic acid follows from the fact that dihydropenicillic acid (IX and X) reacts normally with diazomethane to give a methyl ester containing no nitrogen. This ester reacts with hydrazine hydrate with the loss of 1 mol. each of CH_3OH and H_2O and the formation of a ring compound of structure (XVI).



On heating penicillic acid with hydroxylamine, demethylation and decarboxylation of the acid occur and a compound, possibly the dioxime of $C_6H_8O_2$ (I), is formed, a reaction which would be analogous to the first stage in the reaction with phenylhydrazine. Under the same conditions dihydropenicillic acid and hydroxylamine yield a crystalline product $C_6H_{11}O_3N$ (XVII) which gives no aldoxime or ketoxime reactions. Since it contains all the C atoms of dihydropenicillic acid including the methoxyl group, it almost certainly arises by ring closure between an oxime group formed initially and the carboxyl group, to give an orthoxazine ring structure (XVII).



This reaction is typical of γ -keto-acids and indicates that dihydropenicillie acid can behave as though it possessed such a structure.

Penicillic acid when written in the lactone form (XIII) bears a superficial resemblance to the tetronic acid structure, which has been shown to occur [Clutterbuck *et al.*, 1935] in several metabolic products obtained from *P. Charlesii*. In tetronic acid however the acidity is derived from the hydroxyl group attached to the β -carbon atom, whilst in penicillic acid this group is methylated and cannot therefore be acidic. Hence we must suppose that the acidity of penicillic acid is due to a true carboxyl group obtained by the opening of the lactone ring as indicated in the foregoing argument.

Penicillic acid is almost unique among natural products in that it is an aliphatic open-chain compound, which, although not a methyl ester, yet contains a methoxyl group. The only similar natural products appear to be the sugars digitalose and cymarose. The latter, which occurs in the cardiac glycosides cymarín and periplocymarín, has definitely the constitution



[Elderfield, 1935], which bears some resemblance to penicillic acid. Finally, one other mould metabolic product containing a terminal $=CH_2$ group, *viz.* itaconic acid, has recently been obtained from *Aspergillus itaconicus* by Kinoshita [1931].

EXPERIMENTAL.

*Preparation of penicillic acid.**(a) From P. puberulum Bainier.*

Culture. A subculture of Alsberg and Black's strain of *P. puberulum* Bainier was received in October 1928 from Dr Charles Thom—Ardeer Catalogue No. Ad 113.

35 l. of Raulin medium of the following composition were made up and distributed equally between 100 onc-litre conical flasks: glucose, 75 g.; tartaric acid, 4 g.; NH_4NO_3 , 4 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.6 g.; K_2CO_3 , 0.6 g.; MgCO_3 , 0.4 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.25 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; distilled water, 1500 ml. The flasks were sterilised, inoculated with a spore suspension of the organism and incubated at 24° for 19–23 days. At the end of the incubation period the metabolism solution was filtered, evaporated *in vacuo* to small bulk, acidified and extracted with ether. The crystalline residue remaining after evaporation of the ether was recrystallised 2 or 3 times from hot water + blood charcoal.

The yield of penicillic acid was at first of the order of 5 g. per 100 flasks, but over a period of 3 years the yield fell until in 1931 no penicillic acid could be obtained. In its place a small yield of succinic acid was isolated.

Large colourless rhombic or hexagonal plates, M.P. 64–65°. (Alsberg and Black report the same M.P.) Loss in weight on drying *in vacuo* over P_2O_5 , 9.69, 9.63 %. Theory for $\text{C}_8\text{H}_{10}\text{O}_4 \cdot \text{H}_2\text{O} \rightarrow \text{C}_8\text{H}_{10}\text{O}_4$, 9.58 %. M.P. of dehydrated material, 87°, which is in agreement with Alsberg and Black's figure. Alsberg and Black, however, report that penicillic acid, crystallised from water, contains $2\text{H}_2\text{O}$.

(Found (Schoeller) on anhydrous material: C, 56.39, 56.31; H, 6.09, 6.09 %. Equiv. by titration, 169, 172. $\text{C}_8\text{H}_{10}\text{O}_4$ requires C, 56.44; H, 5.93 %. Equiv. titrating as a monobasic acid, 170. Found OCH_3 17.76 %; $\text{C}_7\text{H}_7\text{O}_5 \cdot \text{OCH}_3$ requires 18.24 %.) The general properties and reactions of the substance, which will be discussed later, are in agreement with those described by Alsberg and Black for penicillic acid, and hence we consider that no reasonable doubt exists as to the identity of the two substances.

(b) From P. cyclopium Westling.

Culture. The culture of *P. cyclopium* used (L.S.H.T.M. Catalogue No. P. 123) was purchased from the Centraalbureau voor Schimmelcultures, Baarn, in August 1931. It was derived from Westling's original strain.

A Raulin-Thom medium was used identical in composition with the Raulin medium used for *P. puberulum* except that 4 g. diammonium tartrate were used in place of 4 g. ammonium nitrate. Cultural conditions were also the same, 10–14-day old cultures on Czapek-Dox agar slopes being employed for inoculation. The flasks were incubated at 24° for 18–23 days. The course of metabolism and details of large scale preparations are summarised in Table I, from which it is evident that this mould has maintained its activity in full for a year.

Although penicillic acid does not appear to be metabolised by this mould, as is evident from a consideration of the bromine absorption figures, the metabolism solutions were worked up when about 0.2 % of glucose still remained, because of the danger of destruction of penicillic acid during evaporation at the alkaline reaction produced after a longer incubation period. Growth of the mould was fairly rapid, a good white surface felt being formed in 5 days which later became much wrinkled and covered with green patches. The reverse was brown and the metabolism solution became progressively darker in colour as incubation continued.

Table I.

Date of inoculation	Incubation period in days	% glucose by polarimeter	p _H	Titrateable acidity ml. N/10 NaOH per 10 ml.	Br absorption mg./ml.
29. x. 34	—	5.35	3.9	2.5	0.10
"	5	4.00	Below 3	5.1	0.72
"	10	1.68	"	6.5	4.96
"	14	1.06	"	5.9	6.4
"	28	Nil	6.2	Negligible	8.8
"	33	Nil	Alkaline	—	8.3
10. xii. 34	23	0.10	4.8	—	7.6
(Average of 100 flasks)					
6. vi. 35	18	0.20	Below 3	—	7.54
(Average of 100 flasks)					
18. ix. 35	20	0.20	"	—	8.0

The metabolism solution from 100 flasks was evaporated *in vacuo* to 500 ml. The penicillic acid crystallising out overnight was collected and recrystallised from 300 ml. boiling water (norite). The crystals, which still contained some potassium hydrogen tartrate, were filtered, washed and completely dried *in vacuo*. They were then extracted with a minimum volume of hot chloroform (100–200 ml.) and 20 volumes of light petroleum were added. The various aqueous filtrates still contained some penicillic acid which could be recovered by extraction with chloroform. Yield of pure hydrated penicillic acid, 73 g. per 100 flasks, corresponding to 52 % of the amount calculated from the bromine absorption figures and 4.2 % of the glucose metabolised.

On crystallisation from light petroleum, B.P. 60–80°, penicillic acid crystallised in needles, M.P. 83–84°, alone or mixed with anhydrous penicillic acid obtained from *P. puberulum*.

(Found (Schoeller): C, 56.46, 56.43; H, 5.93, 5.91; OCH₃, 18.17, 17.82 %. Mol. wt. cryoscopic in dioxan, 176. C₈H₁₀O₄ requires C, 56.44; H, 5.93; OCH₃, 18.24 %. Mol. wt. 170.)

In a Zerewitinoff estimation (Roth) it gave 0.94, 1.09 mols. of CH₄ in pyridine and 0.98, 1.08 mols. in anisole at 18° and 95° in each case respectively.

The above product separated from water in rhombic or hexagonal plates, M.P. 58–64°, alone or mixed with hydrated penicillic acid from *P. puberulum*. Equiv. by titration, 188. C₈H₁₂O₆ requires 188. Mol. wt. cryoscopic in dioxan, 97.5. The hydrated acid is evidently almost completely dissociated into C₈H₁₀O₄ and H₂O in dioxan solution since the theoretical mol. wt. is 188.

In a Zerewitinoff estimation (Roth) it gave 1.91, 2.05 mols. of CH₄ in pyridine, and 1.65, 1.96 mols. in anisole at 18° and 95° in each case respectively.

This strain of *P. cyclopium* yields no penicillic acid when grown on Czapek-Dox medium with glucose as sole source of carbon and NaNO₃ as sole source of nitrogen. Further, a strain of *Penicillium* believed to be *P. cyclopium* Westling, freshly isolated from infected tulip bulbs by our colleague Mr G. Smith, failed to yield penicillic acid even when grown on Raulin-Thom medium.

Penicillic acid is however undoubtedly a product of the metabolism of glucose and not of tartaric acid, since Birkinshaw and Raistrick [1932] obtained it with Alsberg and Black's strain of *P. puberulum* when grown on Czapek-Dox medium containing glucose as the sole source of carbon.

General properties of penicillic acid.

Penicillic acid is soluble in cold water to about 2 %; it is readily soluble in hot water, alcohol, ether, benzene and chloroform and insoluble in cold light petroleum. An aqueous solution just blues Congo red paper and decomposes

carbonates. It gives no colour with aqueous FeCl_3 in the cold and only an orange brown colour on warming. As stated by Alsberg and Black it gives a reddish-purple colour with ammonia on standing. This reaction is fairly delicate since a 0.05 % solution gives a perceptible pink colour with 2-3 vols. of strong ammonia. It gives only a yellow colour with primary amines. It gives no colour with concentrated H_2SO_4 in the cold and chars on heating. It gives no colour with NaNO_2 (γ -methyltetronic acid and certain of its derivatives give a blue colour with this reagent). It gives no colour with Fearon's nitrochromic acid reagent [Fearon and Mitchell, 1932] and hence does not contain a primary or secondary alcoholic group. A neutral aqueous solution of the sodium salt gives only a green precipitate with CuSO_4 solution and no reduction to Cu_2O takes place on boiling. Hence penicillic acid does not contain the $-\text{CHOH}\cdot\text{CO}-$ or $-\text{CO}\cdot\text{CO}-$ grouping.

If penicillic acid is a $\beta\gamma$ -unsaturated lactone it should reduce Tollens's reagent and give an immediate reaction with sodium nitro-prusside [Jacobs and Hoffmann, 1926]. Actually, it reduces ammoniacal silver in $\frac{1}{2}$ -1 hour in the cold, but the reaction with nitroprusside (under the conditions given by Jacobs and Hoffmann, *viz.* in aqueous pyridine solution to which a few drops of dilute caustic soda have been added) is not immediate, but a deep, reddish brown colour develops in a few minutes. An almost immediate deep red colour is however obtained under the following modified conditions: before adding the nitroprusside, the alkaline pyridine solution of penicillic acid is kept for 5 min. or longer. The red colour then obtained with nitroprusside fades to yellow on long standing. Penicillic acid is therefore probably not a $\beta\gamma$ -unsaturated lactone but may be changed into one by the action of alkali. One of the two isomeric structures we have assigned is that of an $\alpha\beta$ -unsaturated lactone.

Ketonic reactions. Neither penicillic acid nor its dihydro-derivative dissolved in 2*N* HCl gives any precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl, except on long standing. When penicillic acid was treated with an excess of hydroxylamine hydrochloride and the solution titrated at intervals with *N*/10 NaOH (bromophenol blue as indicator) 2 equiv. of hydroxylamine were absorbed in 291 hours. The reaction proceeds much more quickly in presence of free hydroxylamine. One mol. of the latter rapidly combines at room temperature, as shown in a number of experiments in which penicillic acid hydrate (0.113 g.) in 5 ml. H_2O was added to 5 ml. of 25 % hydroxylamine hydrochloride and 20 ml. of *N*/10 NaOH. The HCl liberated, as determined by back-titration with *N*/10 HCl, after 10, 20, 30, 60, 180 and 360 min. was 3.7, 5.7, 7.0, 8.4, 10.2 and 10.8 ml. *N*/10 respectively, the theoretical value for the uptake of 1 mol. of hydroxylamine being 6.0 ml. This titration is possible since the sodium salt of penicillic acid behaves as free NaOH towards bromophenol blue. The slow uptake of a second mol. of hydroxylamine is probably due to demethylation with formation of a second keto-group.

In corresponding experiments with dihydropenicillic acid there was no uptake of hydroxylamine during 360 min.

Penicillic acid gives the iodoform reaction with hypoiodite solution.

We have confirmed the observation of Alsberg and Black that penicillic acid is optically inactive.

Derivatives of penicillic acid.

Dihydropenicillic acid. When hydrogenated in aqueous solution using a Pd-norite catalyst, penicillic acid rapidly absorbed 1 mol. of hydrogen (in 2-3 min.) and then gradually absorbed a second mol. in about 7 hours. With the

addition of 2H_2 only non-crystallisable oils were obtained but the addition of 1 mol. of hydrogen gave a homogeneous crystalline product.

Hydrated penicillic acid (5 g.) was dissolved in ethyl alcohol (60 ml.) and about 30 ml. of water were added together with the catalyst prepared from 0.3 g. of PdCl_2 . The mixture was allowed to absorb 596 ml. (corr. N.T.P.) of hydrogen (theoretical for 1 mol.). It was filtered from the catalyst and evaporated to dryness *in vacuo*. The crystalline residue was taken up in ether and treated with 5 volumes of light petroleum (B.P. 50–60°). Colourless needles, 3.7 g., m.p. 86°.

(Found (Schoeller): C, 56.06, 55.93; H, 7.14, 7.02 %. $\text{C}_8\text{H}_{12}\text{O}_4$ requires C, 55.80; H, 7.03 %.)

Dihydropenicillic acid crystallises from water in colourless flat needles, m.p. 62–64°, which contain 1 H_2O . (Found: loss on drying *in vacuo* over concentrated H_2SO_4 , 9.64 %. $\text{C}_8\text{H}_{12}\text{O}_4 \cdot \text{H}_2\text{O}$ requires H_2O , 9.48 %.) The anhydrous substance so obtained melts at 83–85°.

Dihydropenicillic acid hydrate is less soluble in cold water than penicillic acid hydrate and is a much weaker acid, having no action on Congo red paper but turning blue litmus red.

Penicillic acid dibromide. A solution of Br (2.5 g. \equiv 2 atoms Br) in glacial acetic acid (100 ml.) was slowly added at room temperature to a solution of hydrated penicillic acid (2.834 g.) in the same solvent (30 ml.). The reaction, slow at first, later became very rapid. No HBr was evolved. The mixture was evaporated to dryness in a vacuum desiccator over solid KOH. Yield almost theoretical, m.p. 154°. Recrystallised from CCl_4 , colourless slender needles, m.p. 154–5°.

(Found (Schoeller): C, 29.25, 29.24; H, 3.19, 3.20; Br, 48.85, 48.80 %. Equiv. by titration 326. $\text{C}_8\text{H}_{10}\text{O}_4\text{Br}_2$ requires C, 29.10; H, 3.05; Br, 48.44 %. Equiv. (monobasic) 330.)

On bromination in aqueous or 50 % aqueous acetic acid solution penicillic acid absorbs four atoms of Br, but complex reactions take place and result in partial breakdown.

Acetylpenicillic acid. Hydrated penicillic acid (0.5 g.), anhydrous sodium acetate (1.0 g.) and acetic anhydride (2 ml.) were heated together in a bath at 140° for 10 min. and the product was poured into water. An oil separated. After 24 hours the product was extracted with ether, the ether removed and the residue dried *in vacuo* over KOH and H_2SO_4 . On standing it set to a crystalline mass, which was recrystallised twice from light petroleum.

Colourless elongated prisms, m.p. 72°. (Found (Schoeller): C, 56.81, 56.68; H, 5.95, 5.72; OMe, 14.01, 14.18 %. $\text{C}_{10}\text{H}_{12}\text{O}_5$ requires C, 56.58; H, 5.71; OMe, 14.63 %.) The product is therefore a monoacetyl derivative of anhydrous penicillic acid.

Action of diazomethane on penicillic acid.

(a) *The pyrazoline methyl ester of penicillic acid (XIV)*. Anhydrous penicillic acid (1 g.) was treated with excess of diazomethane in ether. A vigorous effervescence occurred. After removal of the ether the residue set to a mass of colourless crystals (prisms) which were recrystallised from light petroleum (50–60°); m.p. 74°, not raised on further recrystallisation; yield 1.0 g.

(Found (Schoeller): C, 53.26, 53.04; H, 6.21, 6.03; N, 12.29, 12.20; macro-Zeisel, OCH_3 , 27.25, 27.29 %. $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_2$ requires C, 53.08; H, 6.24; N, 12.40; 2OCH_3 , 27.45 %.)

(b) *Pyridazone (XV)*. The pyrazoline ester (XIV) (0.5 g.) in ethyl alcohol (2 ml.) was treated with 0.5 ml. of 50 % aqueous hydrazine hydrate. Considerable

heat was evolved. Colourless crystals separated overnight, 0.15 g., M.P. 174° (decomp.). After recrystallisation from alcohol they appeared to disintegrate without sintering at 175° and melted at 181° with decomposition.

(Found (Schoeller): C, 51.77, 51.87; H, 5.88, 5.78; N, 27.25, 27.19; OCH_3 , 14.71, 14.87%. $\text{C}_9\text{H}_{12}\text{O}_2\text{N}_4$ requires C, 51.88; H, 5.81; N, 26.92; one OCH_3 , 14.90%.)

(c) *Phenylpyridazone* (XV). The pyrazoline ester (XIV) (0.6 g.) was heated with phenylhydrazine hydrochloride (0.9 g.) and 1 drop of conc. HCl in a few ml. of alcohol for about 15 min. on the water-bath. There was some action, indicated by the production of a yellow colour. On dilution of a test sample with water practically all dissolved. Therefore 1 g. of crystalline sodium acetate was now added and the heating was continued for 15–20 min. longer. On cooling, 0.58 g. of somewhat dark-coloured crystalline material was obtained, which on warming with about 30 ml. of alcohol partly dissolved, yielding a greenish solution changing to brown. A lemon-yellow crystalline substance remained undissolved. This was recrystallised from alcohol in which it was not very soluble. It was observed that on boiling with alcohol, the solution, at first lemon-yellow in colour, rapidly became emerald-green. The recrystallised material had M.P. 198–199°.

(Found (Schoeller): C, 63.06, 63.21; H, 5.59, 5.67; N, 19.84, 19.94; OCH_3 , 10.53, 10.34%. $\text{C}_{15}\text{H}_{16}\text{O}_2\text{N}_4$ (1 MeO) requires C, 63.34; H, 5.68; N, 19.72; OCH_3 , 10.91%.)

Action of diazomethane on dihydropenicillic acid.

(a) *Dihydropenicillic acid methyl ester*. Dihydropenicillic acid (0.65 g.) was treated with excess of diazomethane in ether for several hours. The solution was filtered and the ether evaporated. The product (0.68 g.) was a colourless, somewhat viscid oil with an ester-like smell. It was insoluble in H_2O but readily soluble in cold light petroleum. It contained no nitrogen. A Zeisel determination on the material after drying *in vacuo* gave 32.24% OCH_3 . Calc. for $\text{C}_9\text{H}_{14}\text{O}_4$ (2 methoxyl), 33.32%. The material is therefore a true methyl ester of dihydropenicillic acid.

(b) *Action of hydrazine hydrate on the above ester*. The methyl ester (0.33 g.), alcohol (1.0 ml.) and 50% hydrazine hydrate (0.40 ml.) were mixed and kept for several hours. Crystals (0.16 g.) separated. These were collected and washed with a little alcohol and recrystallised from absolute alcohol. Colourless prisms, M.P. 207–208°, of structure XVI.

(Found (Schoeller): C, 57.05, 57.22; H, 7.12, 7.15; N, 16.86, 16.75; macro Zeisel OCH_3 , 19.02%. $\text{C}_8\text{H}_{12}\text{O}_2\text{N}_2$ requires C, 57.11; H, 7.20; N, 16.66; OCH_3 , 18.45%.)

Action of dimedon on penicillic acid.

Penicillic acid (1 g.), dimedon (2 g.) and anhydrous sodium acetate (2 g.) were mixed with H_2O (25 ml.). The mixture, which gave a clear solution on warming, was heated at 100° for 1½ hours. An oil soon separated which crystallised on keeping. The product was recrystallised by solution in alcohol and cautious addition of water. M.P. 201–203°, with decomposition at 204°.

(Found (Weiler): C, 62.20, 62.33; H, 7.24, 7.41; macro-Zeisel, OCH_3 , 10.09%. $\text{C}_{16}\text{H}_{22}\text{O}_6$ requires C, 61.89; H, 7.15; 1 OCH_3 , 10.00%.) The product is thus formed by combination of 1 mol. of (anhydrous) penicillic acid, $\text{C}_8\text{H}_{10}\text{O}_4$ with 1 mol. of dimedon, $\text{C}_8\text{H}_{12}\text{O}_2$. It is not the condensation typical of aldehydes in which 2 mols. of dimedon are employed, and 1 mol. of water is lost.

Action of hydroxylamine on penicillic acid.

Hydrated penicillic acid (5 g.) was heated with hydroxylamine hydrochloride (5 g.) anhydrous sodium acetate (10 g.) and water (50 ml.) for 75 min. in boiling water. Colourless needles separated overnight, 2.35 g.; m.p. 205°, raised to 210–212° (decomp.), after recrystallisation from alcohol.

(Found (Weiler): C, 50.94, 50.99; H, 6.72, 6.78; N, 19.63, 19.65 %. $C_6H_{10}O_2N_2$ requires C, 50.66; H, 7.09; N, 19.72 %.) This would correspond with a dioxime of $C_6H_8O_2$ (I of introduction), derived by the decarboxylation and demethylation of penicillic acid.

Action of hydroxylamine on dihydropenicillic acid.

Dihydropenicillic acid (3 g.) was heated at 100° for 2 hours with hydroxylamine hydrochloride (3 g.), anhydrous sodium acetate (6 g.) and water (50 ml.). An oil separated which was extracted with ether. The ether solution was washed with aqueous sodium bicarbonate and was evaporated. The residue set to a solid crystalline mass which was again dissolved in a little ether and chilled rapidly by a current of air. The crystals separating were collected and washed with chilled ether. m.p. 54–56°.

(Found (Weiler): C, 56.78; H, 6.41; N, 8.21; OCH_3 , 18.09 %. $C_8H_{11}O_3N$ requires C, 56.76; H, 6.56; N, 8.28; OCH_3 , 18.34 %.) The product gave no reaction for aldoxime or ketoxime by oxidation with monopersulphuric acid and was not attacked by acetic anhydride or by PCl_5 suspended in ether. Hence it does not contain a hydroxyl group. It probably has the structure XVII.

Action of phenylhydrazine on penicillic acid. Investigation of resulting product.

Hydrated penicillic acid (20 g.) and crystallised sodium acetate (30 g.) were dissolved by gentle warming in 100 ml. of water. Phenylhydrazine (25 g.) and glacial acetic acid (15 ml.) were then added. A vigorous reaction occurred with considerable rise in temperature and much CO_2 was evolved. The reaction appeared to be complete in about 5 min. and a yellow tarry mass separated. After about an hour the aqueous liquid was poured off, the tar was washed once with water which was poured off and then 50 ml. of absolute alcohol were added. On warming, the whole of the tarry mass disappeared and was replaced by yellow crystals. The mixture was cooled and the crystals were collected and washed with a little alcohol. Yield, 13.5–14 g. of almost pure product. Yellow prisms, m.p. 176° (Alsberg and Black [1913] give 171°).

(Found (Schoeller): C, 73.96, 73.76; H, 6.89, 6.71; N, 19.21, 19.13 %. $C_{18}H_{20}N_4$ requires C, 73.94; H, 6.90; N, 19.17 %.)

The substance is a phenylpyrazoline phenylhydrazone (II of introduction) of $C_6H_8O_2$ (I). It gives an intense purple colour with H_2SO_4 and $K_2Cr_2O_7$ (Knorr's reaction for pyrazolines), and also with 50 % H_2SO_4 + aqueous $FeCl_3$. With H_2SO_4 alone it gives an intense green colour.

(a) *Oxidation of the pyrazoline (II) to a pyrazole (III).* 7 g. of (II) were dissolved by warming in glacial acetic acid (175 ml.) and the solution was cooled rapidly to 40° (incipient crystallisation). Lead dioxide (15 g.) was gradually added, the flask being well shaken and cooled after each addition so that the temperature did not rise appreciably above 40°. During the addition of PbO_2 the colour of the solution changed to a dark green, later becoming brown.

The solution was treated with 175 ml. of 50 % (by volume) H_2SO_4 and left overnight. Next day 350 ml. of water were added and the lead sulphate was

removed by filtration. The filtrate was treated with 500 ml. of water and chilled. Colourless crystals separated, 0.76 g., M.P. 67°. The solution was made slightly alkaline and extracted with ether. The ether extract (0.77 g.) crystallised on keeping. A further 2.65 g. of syrup, later crystallising, was obtained by extraction with ether of the lead sulphate precipitate. The three products were combined, recrystallised from light petroleum and sublimed *in vacuo* giving colourless crystals, M.P. 72–74°.

(Found (Weiler): C, 72.02; H, 5.83; N, 14.14%. $C_{12}H_{12}ON_2$ requires C, 71.97; H, 6.08; N, 14.01%.) This corresponds with a phenylpyrazole derived from the substance $C_6H_8O_2$ (I) and phenylhydrazine. One phenylhydrazine group, obviously present as a phenylhydrazone, has been removed by hydrolysis from (II), and the pyrazoline ring has been oxidised to a pyrazole ring to give (III). (III) gave a strong Zimmermann reaction for the $\cdot CO \cdot CH_2$ group but gave no Schiff or Angeli-Rimini reaction for aldehydes. On the other hand it readily yielded an oxime. The oxygen is therefore present as a keto-, not as an aldehyde, group.

(b) *Preparation of the oxime of the pyrazole (III).* 0.64 g. of (III) was dissolved in alcohol (5 ml.) and hydroxylamine hydrochloride (0.25 g.) and crystalline sodium acetate (0.5 g.) were added in 3 ml. of water. The clear solution almost immediately began to deposit crystals. The mixture was warmed on the water-bath for 20 min. The crystals obtained on cooling were collected and washed with 2 ml. of 50% aqueous alcohol: yield 0.49 g.; M.P. 149° not raised on sublimation *in vacuo*.

(Found (Weiler): C, 67.03; H, 6.05; N, 19.41%. $C_{12}H_{13}ON_3$ requires C, 66.95; H, 6.09; N, 19.54%.)

The only possible substituents of the pyrazole ring (apart from the 1-phenyl group), assuming that a keto-group is present, are $\cdot CO \cdot CH_2 \cdot CH_3$, $\cdot CH_2 \cdot CO \cdot CH_3$ or $\cdot CO \cdot CH_3 + \cdot CH_3$. The first of these is excluded by the fact that (III) is readily oxidised by hypiodite solution, with production of iodoform, to a pyrazole-carboxylic acid (IV) having one carbon atom less than the original pyrazole (III).

(c) *Oxidation of the pyrazole (III) with hypiodite to a monocarboxylic acid (IV).* The sublimed pyrazole (0.44 g.) was dissolved in methyl alcohol (500 ml.). Water (500 ml.) was added, followed by $N/10$ iodine (500 ml.) and $N/10$ NaOH (600 ml.). The mixture was left overnight. The crystalline iodoform was removed by filtration and the filtrate was acidified with $2N$ H_2SO_4 (50 ml.). Thiosulphate solution was then added to remove all free iodine. The solution was extracted with ether and the ether solution shaken with aqueous Na_2CO_3 , which was then acidified and extracted with ether. On removal of the solvent 0.43 g. acid was obtained, M.P. after sublimation 170°.

(Found (Weiler): C, 65.22; H, 4.98; N, 13.78%. $C_{11}H_{10}O_2N_2$ (IV) requires C, 65.31; H, 4.99; N, 13.87%.)

(d) *Oxidation of (IV) with alkaline $KMnO_4$ to give 1-phenylpyrazole-3:4-dicarboxylic acid (V).* 0.7 g. of (IV) was neutralised with aqueous $N/10$ NaOH and to the hot solution was added, drop by drop, $KMnO_4$ (1.5 g.) dissolved in water (200 ml.). The mixture was heated at 100°. The precipitated MnO_2 was separated by filtration and the filtrate acidified. An immediate precipitate (0.26 g.) proved to be unchanged (IV), which was separated. The filtrate on evaporation *in vacuo* yielded impure (V) mixed with a little (IV), which was removed by fractional vacuum sublimation. (V) was finally crystallised from water to give colourless elongated plates, M.P. 232°.

(Found (Schoeller): C, 56.98, 56.97; H, 3.67, 3.62; N, 12.09, 11.93%. Equiv. by titration (end-point not sharp to phenolphthalein), 127–118. $C_{11}H_8O_4N_2$ (V) requires C, 56.87; H, 3.47; N, 12.07%. Equiv. 116.)

The acid (V) gave no depression in M.P. when mixed with synthetic 1-phenylpyrazole-3:4-dicarboxylic acid (recorded M.P. 234°; Balbiano [1898]).

Synthesis of 1-phenylpyrazole-3:4-dicarboxylic acid. 1-Phenyl-3:4-dimethylpyrazole (2.2 g.) obtained from 1-phenyl-3:4-dimethylpyrazolone by the method of Stoermer and Martinsen [1907] was heated with KMnO_4 (13 g.) in water (400 ml.) under reflux at 100°. When all the KMnO_4 was reduced, the solution was filtered and evaporated on the water-bath to small volume. The dicarboxylic acid, mixed with 1-phenyl-3-methylpyrazole-4-carboxylic acid was separated by acidification and was purified by fractional vacuum sublimation and crystallisation from a mixture of ethyl acetate and light petroleum: colourless plates, M.P. 232° alone or mixed with the product (V) from penicillic acid. (Found (Schoeller): C, 56.85; H, 3.47; N, 12.32%. $\text{C}_{11}\text{H}_8\text{O}_4\text{N}_2$ requires C, 56.87; H, 3.47; N, 12.07%.) *Dimethyl ester.* The product obtained by the action of diazomethane on the above acid was recrystallised from ether-light petroleum: colourless plates, M.P. 89° alone or mixed with the corresponding product from penicillic acid.

(e) *Decarboxylation of 1-phenyl-4-methylpyrazole-3-carboxylic acid (IV) to give 1-phenyl-4-methylpyrazole (VI).* The acid (IV) (0.4 g.) was heated under reflux (air condenser) in a bath at 250–260° for 30 min. after which no more evolution of gas could be observed. On cooling the residue set solid. This was taken up in ether, washed with aqueous sodium carbonate and the ether was evaporated. The crystalline residue weighed 0.28 g. This was dissolved in light petroleum (B.P. 40–50°) in which it was extremely soluble. It was crystallised by cooling in solid CO_2 . The substance had M.P. 43°, and analysed correctly for 1-phenyl-4-methylpyrazole. (In the literature this substance is given as a liquid.)

(Found (Schoeller): C, 75.78; H, 6.29; N, 17.83%. $\text{C}_{10}\text{H}_{10}\text{N}_2$ (VI) requires C, 75.90; H, 6.37; N, 17.72%.)

(f) *Oxidation of 1-phenyl-4-methylpyrazole (VI) with KMnO_4 to 1-phenylpyrazole-4-carboxylic acid (VII).* 0.16 g. of (VI) was oxidised at 100° with 0.5 g. (50% excess) of KMnO_4 dissolved in water. When all the KMnO_4 had disappeared there was still unchanged (VI) present. The solution was filtered from manganese dioxide and unchanged material and was acidified. The crystalline precipitate was collected, 0.05 g.; M.P. on sublimed sample, 219–221°.

(Found (Schoeller): C, 63.96; H, 4.38; N, 14.69%. Equiv. by titration = 190. $\text{C}_{10}\text{H}_8\text{O}_2\text{N}_2$ (VII) requires C, 63.80; H, 4.29; N, 14.89%. Equiv. = 188.)

Action of HI on penicillic acid.

Anhydrous penicillic acid (5 g.) was heated with HI (sp. gr. 1.7, 50 ml.) and red phosphorus (2 g.) for 2½ hours under an air condenser; bath temperature 160°. The cooled reaction mixture was diluted, extracted with ether, free iodine removed by shaking with aqueous thiosulphate, and the ether solution evaporated. The residue was heated for 45 min. with zinc (20 g.) and 6*N* HCl (50 ml.) under reflux to remove combined iodine and then extracted with ether. Weight 1.10 g. of an oil which was neutralised with 6.25 ml. *N* NaOH and converted into the *p*-phenylphenacyl ester by heating with water (10 ml.), alcohol (30 ml.) and *p*-phenylphenacyl bromide (1.72 g.): colourless needles from light petroleum M.P. 63°.

(Found (Schoeller): C, 74.64, 74.74; H, 6.58, 6.65%. OCH_3 , nil. $\text{C}_{21}\text{H}_{22}\text{O}_4$ requires C, 74.50; H, 6.56%.)

This represents the *p*-phenylphenacyl ester of an acid $\text{C}_7\text{H}_{12}\text{O}_3$ which in the light of the results obtained on the action of HI on dihydropenicillic acid (see next section) is believed to be $\text{CH}_3(\text{CH}_2)_2\text{C}\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$.

Action of HI on dihydropenicillic acid.

Dihydropenicillic acid (20 g.) was heated with HI (sp. gr. 1.7, 150 ml.) and red phosphorus (8 g.) for 2½ hours. The details of the isolation of the crude reduction product were the same as in the previous section.

The product was then divided into acid, lactone and neutral fractions. By shaking the ether solution with aqueous Na_2CO_3 the acids were removed. They were regenerated by acidification of the aqueous layer and weighed 1.71 g. The ethereal layer was evaporated and the product was heated for 30 min. on the water-bath with an excess of *N* NaOH. The neutral substances extracted by ether weighed 0.27 g. The lactones were regenerated by acidification of the aqueous layer and extracted with ether: weight 2.2 g. 2 g. of the lactones were distilled at ordinary pressure and yielded a trace of water and 0.86 g. of liquid distilling at 217–218°. There was a considerable amount of higher-boiling residue. The oil, b.p. 217–218°, was converted into the phenylhydrazide by heating under reflux with 0.75 g. of phenylhydrazine in 2–3 ml. of absolute alcohol for about 3 hours. The product yielded crystalline plates on keeping exposed to the air. These were treated with a little ether, collected and washed with ether and recrystallised by solution in a small amount of absolute alcohol and addition of several volumes of ether: plates, m.p. 126°. There was no depression in m.p. when this material (structure VIII) was mixed with synthetic γ -hydroxy- δ -methylhexanoic acid phenylhydrazide (*v. infra*).

(Found (Schoeller): C, 65.77, 65.94; H, 8.46, 8.64; N, 12.54%. $\text{C}_{13}\text{H}_{20}\text{O}_2\text{N}_2$ requires C, 66.07; H, 8.50; N, 11.87%.)

Synthesis of γ -hydroxy- δ -methylhexanoic acid phenylhydrazide. Ethyl $\delta\delta$ -dicarboethoxy- β -keto- $\alpha\alpha$ -dimethylvalerate (20 g., b.p. 150°/1 mm.; cf. Conrad [1897]) was heated with 2*N* H_2SO_4 (250 ml.) under reflux, in an oil bath at 120°. The CO_2 was driven out by a current of CO_2 -free air and absorbed in potash bubblers and weighed. The hydrolysis was practically complete in 24 hours. The product was extracted with ether and transferred to sodium carbonate solution. It was liberated by HCl and again extracted with ether. After removal of the ether it set to a solid mass of crystals, weight 9.3 g. This material dissolved in alcohol (70 ml.) was heated to boiling, and sodium (15 g.) was added during 10 min. [cf. Losanitsch, 1914]. Then 130 ml. of alcohol were gradually added so as to dissolve the remainder of the sodium in 20–30 min. The solution was cooled and treated with 130 ml. of water and alcohol was removed *in vacuo*. The residue was treated with 60 ml. of a mixture of 50 g. of sulphuric acid and 50 ml. of water (total volume of mixture 72 ml.). The product was boiled for 5 min., cooled and extracted with ether. The oil (7.15 g.) on distillation gave 6.35 g. of product of b.p. 227–230° at atmospheric pressure.

The lactone (3.25 g.), phenylhydrazine (2.7 g.) and alcohol (5 ml.) were heated together on a steam-bath under reflux for 2 hours. The product was cooled and treated with much ether. It gave 1.16 g. of crystals which, after recrystallisation from alcohol-ether, appeared as glistening plates, m.p. 126° alone or mixed with the corresponding compound from penicillic acid.

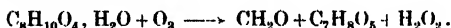
Oxidation of penicillic acid by ozone. Production of formaldehyde and a keto-acid $\text{C}_7\text{H}_8\text{O}_5$.

Ozonised oxygen was passed into a solution of hydrated penicillic acid (0.994 g.) in water (55 ml.) at 0–5° for 5–6 hours. A considerable amount of unchanged acid crystallised out during the experiment. The mixture was kept overnight, and a test on the filtered solution showed that an aldehyde was present

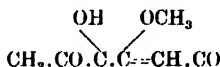
(colour restored to Schiff's reagent in less than 2 min.). An equal volume of Brady's reagent was added to the bulk of the solution, and after several hours the yellow crystalline precipitate was collected, washed, dried (0.2 g.), triturated with saturated Na_2CO_3 and filtered. The filtrate was only pale red in colour and gave no appreciable precipitate on acidification, but by repeated washing of the insoluble residue with water, a deep red solution was obtained which gave a good yellow precipitate (0.10 g.) on acidification with conc. HCl . The acidic hydrazone crystallised from benzene-light petroleum in groups of small, flat, pointed, yellow needles, m.p. $202\text{--}204^\circ$ with some effervescence.

(Found (Weiler): C, 44.35; H, 3.58; N, 15.77; OCH_3 , 8.93%. $\text{C}_{13}\text{H}_{12}\text{O}_8\text{N}_4$ (monodinitrophenylhydrazone of $\text{C}_7\text{H}_8\text{O}_5$) requires C, 44.31; H, 3.44; N, 15.91; OCH_3 , 8.81%.)

The residual dinitrophenylhydrazone insoluble in dilute Na_2CO_3 was crystallised from boiling water in which it was almost completely soluble. It formed slender, brownish yellow needles, m.p. $162\text{--}164^\circ$. Mixed with an authentic specimen of formaldehyde dinitrophenylhydrazone (m.p. $164\text{--}165^\circ$) it melted at $162\text{--}165^\circ$. The action of ozone on penicillic acid therefore proceeds according to the following equation:



It was desirable to determine whether the compound $\text{C}_7\text{H}_8\text{O}_5$ was an aldehydo- or keto-acid. To this end, the ozonisation experiment was repeated and formaldehyde removed from the resulting solution by repeated evaporation to small volume *in vacuo* at $35\text{--}40^\circ$. Eventually, a solution was obtained which, when made up to one-half of the original volume, did not restore the colour to Schiff's reagent, even on long standing, but gave a good precipitate of an acidic dinitrophenylhydrazone with Brady's reagent, which proved to be identical with the acidic dinitrophenylhydrazone described above. The oxidation product $\text{C}_7\text{H}_8\text{O}_5$ may therefore be the lactone of a keto-acid, *i.e.*



(Found on dinitrophenylhydrazone: equiv. by micro-titration, 318. $\text{C}_{13}\text{H}_{12}\text{O}_8\text{N}_4$ titrating as a monobasic acid requires equiv. 352.)

Oxidation of dihydropenicillic acid by aqueous potassium permanganate.

Formation of oxalic acid and the methyl ester of dimethylpyruvic acid.

To a solution of dihydropenicillic acid (0.77 g.) in water (100 ml.) at 0° , was slowly added during 3 hours $N/10$ KMnO_4 (300 ml.) with stirring and cooling in ice. The liquid was kept for 30 min., after which the supernatant liquid was quite colourless. The oxidation was evidently not yet complete although 2 atoms of available oxygen had been used up. After filtration from MnO_2 , the filtrate, which was practically neutral to litmus, was divided into two equal parts, to one of which was added an equal volume of Brady's reagent, and to the other, after making slightly alkaline to phenolphthalein, an excess of $N/2$ CaCl_2 . The dinitrophenylhydrazone so obtained weighed 0.38 g. (55% of theoretical, assuming that it is derived from $\text{C}_6\text{H}_{10}\text{O}_5$), whilst the precipitate of Ca oxalate weighed only 0.035 g., *i.e.* only 12% of the theoretical for the expected formation of 1 mol. of oxalic acid. The oxalic acid was isolated by repeated extraction of a solution of the Ca salt in HCl with ether, removal of the solvent, and sublimation

of the residue at 80–90° in a high vacuum. The crystalline sublimate melted at 189–190° alone or mixed with authentic anhydrous oxalic acid.

The dinitrophenylhydrazone, which was insoluble in Na_2CO_3 solution, was purified by repeated crystallisation from light petroleum (B.P. 80–100°) in which it is sparingly soluble in the cold, but moderately so at the B.P. It formed fine flat, yellow needles and prisms, m.p. 176–178°, without decomposition.

(Found (Schoeller): C, 46.42, 46.39; H, 4.72, 4.55; N, 17.84, 17.94; OCH_3 , 10.01, 9.99%. $\text{C}_{12}\text{H}_{14}\text{O}_6\text{N}_4$ (monodinitrophenylhydrazone of $\text{C}_6\text{H}_{10}\text{O}_3$) requires C, 46.44; H, 4.55; N, 18.06; OCH_3 , 10.00%.) When triturated with *N* NaOH it slowly dissolved to give a deep brown solution, which yielded on acidification an acidic dinitrophenylhydrazone, crystallising from aqueous alcohol in yellow needles, m.p. 196–197° (decomp.), alone or mixed with the authentic dinitrophenylhydrazone of dimethylpyruvic acid, prepared by the method of Ramage and Simonsen [1935]. (These authors quote m.p. 194–195° for the hydrazone.)

The synthetic dimethylpyruvic acid (1.2 g.) was also esterified by excess of ethereal diazomethane. The solvent was removed and the residual oil was taken up in water (500 ml.) and the crude hydrazone thrown down by addition of Brady's reagent was purified by trituration with *N* NaOH for a few min., and finally by crystallisation from light petroleum; yellow needles and prisms, m.p. 178–180°. Mixed with the dinitrophenylhydrazone of the oxidation product of dihydropenicillic acid the m.p. was 176–179°.

Action of ozone on dihydropenicillic acid.

Methyl dimethylpyruvate was also produced when ozonised O_2 was passed into a solution of dihydropenicillic acid (0.8 g.) in water (50 ml.) at 0–10° for 12 hours. Absorption of O_3 was very slow indeed, even although the gas was distributed through a sintered glass plate. The resulting liquid did not restore the colour to Schiff's reagent even on long standing, but gave a slight yellow precipitate with Brady's reagent which proved to be absolutely insoluble in aqueous Na_2CO_3 . Hence glyoxylic acid is not present in the solution. After crystallisation from light petroleum, the dinitrophenylhydrazone melted at 178–180° alone or mixed with the authentic dinitrophenylhydrazone of methyl dimethylpyruvate. The expected glyoxylic acid had evidently been oxidised to oxalic acid, since the neutralised reaction liquid gave a slight precipitate with CaCl_2 solution, insoluble in acetic acid, but soluble in nitric acid.

Acid hydrolysis of penicillic acid.

Hydrated penicillic acid (0.4921 g.) was heated with 50 ml. of boiling 2*N* H_2SO_4 under reflux in a stream of nitrogen. The gaseous products were bubbled through standard baryta solution. Evolution of CO_2 ceased after 24 hours and was then equivalent to 0.0327 g. of C or 12.49 g. per g. mol. of penicillic acid. Thus 1 mol. of penicillic acid yields 1 mol. of CO_2 on prolonged acid hydrolysis. The reaction mixture was found by titration to contain no acid other than the mineral acid added. The only product of acid hydrolysis of 10 g. penicillic acid other than CO_2 which was isolated was obtained as a 2:4-dinitrophenylhydrazine derivative. It formed orange-brown crystals from aqueous dioxan, m.p. 254°.

(Found: C, 46.84; H, 4.19; N, 19.74%. $\text{C}_{11}\text{H}_{12}\text{O}_5\text{N}_4$ requires C, 47.12; H, 4.32; N, 20.00%.) This corresponds to the mono-dinitrophenylhydrazone of $\text{C}_6\text{H}_8\text{O}_2$ or the bis-dinitrophenylhydrazone of $\text{C}_{10}\text{H}_{16}\text{O}_4$. It gave a positive Neuberg's test for bis-dinitrophenylhydrazones (a purple colour on addition of KOH to its alcoholic solution). The parent substance is therefore $\text{C}_{10}\text{H}_{16}\text{O}_4$, possibly formed

by condensation of 2 mols. of acetylpropionyl, $C_6H_8O_2$, which as will be shown later is a product of the alkaline hydrolysis of penicillic acid.

The same product was obtained on hydrolysis of penicillic acid with boiling aqueous sodium acetate solution.

Acid hydrolysis of dihydropenicillic acid.

Dihydropenicillic acid (2 g.) was heated with 80 ml. of boiling 2*N* H_2SO_4 under reflux in a stream of nitrogen. 1 mol. of CO_2 was evolved in 20 hours. The volatile products were bubbled through Brady's reagent before absorption of CO_2 and from the precipitated 2:4-dinitrophenylhydrazones (2 g. crude) there were isolated three different products:

(a) Red prisms *ex* benzene, m.p. 226–227° (decomp.); insoluble in light petroleum (b.p. 60–80°). Gave a positive Neuberg's test for bis-dinitrophenylhydrazones. (Found (Schoeller) after drying at 110° in high vacuum: C, 45.62, 45.63; H, 3.92, 3.87; N, 23.31 %. $C_{18}H_{18}O_8N_8$ requires C, 45.55; H, 3.83; N, 23.63 %.)

(b) Golden needles *ex* light petroleum and finally from ether, m.p. 186–187°. Insoluble in boiling H_2O . Negative Neuberg's test. (Found (Schoeller): C, 48.91; H, 4.79; N, 19.03 %. $C_{12}H_{14}O_8N_4$ requires C, 48.97; H, 4.80; N, 19.08 %.)

(c) Lemon-yellow rhombic plates *ex* boiling water, light petroleum and ether. m.p. 121°. Negative Neuberg's test. (Found (Schoeller): C, 49.36, 49.28; H, 4.76, 4.89; N, 18.99 %. $C_{12}H_{14}O_8N_4$ requires C, 48.97; H, 4.80; N, 19.08 %.)

Hence (a) is the bis-dinitrophenylhydrazone, and (b) and (c) are the mono-derivatives of $C_6H_{10}O_2$, which from a consideration of the formula established for dihydropenicillic acid (IX and X) we believe to be acetyl*isobutyryl*, arising from the parent substance by decarboxylation and demethylation. Acetyl*isobutyryl*, with limited amounts of Brady's reagent could give rise to a mixture of one bis- and two different mono-dinitrophenylhydrazones.

Alkaline hydrolysis of penicillic acid.

Alsberg and Black [1913] record the formation (but not the analysis) of "delicate needles" when penicillic acid is treated with boiling aqueous baryta. This experiment was repeated. Penicillic acid (5 g.) was heated with *N*/4 baryta (500 ml.) under reflux in a current of nitrogen for 4 hours. The gaseous products were bubbled through Brady's reagent. A complex mixture of hydrolysis products was formed, and the following were isolated, mostly as derivatives either from the Brady's reagent or from the hydrolysis solution.

(1) CO_2 .

(2) A substance $C_5H_{10}O_2$ isolated as the mono-dinitrophenylhydrazone: yellow needles *ex* alcohol, m.p. 193–194°. (Found (Schoeller): C, 46.63; H, 4.97; N, 19.99 %. Mol. wt. 274, 282. $C_{11}H_{14}O_8N_4$ requires C, 46.80; H, 5.00; N, 19.86 %; mol. wt. 282.) The constitution of this hydrolysis product is unknown but it was shown not to be either β -keto- γ -hydroxypentane or β -hydroxy- γ -ketopentane as described in the literature.

(3) Acetylpropionyl, $C_6H_8O_2$, isolated as the bis-dinitrophenylhydrazone: red crystals *ex* benzene. Insoluble in boiling alcohol. Gave positive Neuberg's test; m.p. 272–274° (decomp.). (Found (Schoeller): C, 44.87; H, 3.62; N, 24.84 %; mol. wt. 372, 364. $C_{17}H_{16}O_8N_8$ requires C, 44.32; H, 3.50; N, 24.35 %; mol. wt. 460.) Gave no depression on mixing with a synthetic specimen prepared from diethylketone by the method of Diels and Stephan [1907].

(4) A substance $C_8H_{12}O$ isolated as the monodinitrophenylhydrazone; dark red needles *ex* alcohol, m.p. 152–154°. (Found (Schoeller): C, 55.32; H, 5.14;

N, 18.83%; mol. wt. 266, 279. $C_{14}H_{16}O_4N_4$ requires C, 55.24; H, 5.30; N, 18.43%; mol. wt. 304.) This substance probably arises by condensation of some of the hydrolysis products.

(5) A substance $C_{10}H_{10}O_2$, colourless irregular prisms, m.p. 98–102°, probably identical with the “delicate needles” of Alsberg and Black. (Found (Schoeller): C, 74.20, 74.11; H, 6.39, 6.43%; mol. wt. 154, 159. $C_{10}H_{10}O_2$ requires C, 74.05; H, 6.22%; mol. wt. 162.) The substance titrates as a lactone but sufficient was not available for an accurate titration value. It is obviously a secondary condensation product.

(6) Methyl alcohol identified as the *p*-nitrobenzoate.

(7) Formic acid isolated as the lead salt. (Found: Pb, 69.2%. $Pb(H.CO_2)_2$ requires Pb, 69.7%.)

SUMMARY.

Penicillic acid, $C_8H_{10}O_4$, a metabolic product of *Penicillium puberulum* Bainier, is also formed in considerably larger yield by *P. cyclopium* Westling. The study of its reactions and breakdown products establishes its constitution as γ -keto- β -methoxy- δ -methylenic- Δ^4 -hexenoic acid or the corresponding γ -hydroxylactone.

We tender our best thanks to the Research Council of Imperial Chemical Industries, Ltd., for a grant to one of us (J. H. B.).

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LXV. A STUDY OF THE RELATIVE AMOUNTS OF THE PROTEIN AND NON-PROTEIN NITROGENOUS CONSTITUENTS OCCURRING IN PASTURE HERBAGE, AND THEIR SIGNIFICANCE IN THE GRAZING OF THE HERBAGE BY STOCK.

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INTRODUCTION.

IN a comprehensive study [Greenhill, 1930, 1] of the chemical composition of pasture herbage as grazed under the intensive system of grass-land management, extending over the seasons 1926 to 1929, it was found that, of the total nitrogen of the herbage, the proportion present in protein form (as determined by the Stützer method) was more or less constant under all conditions. The value ranged usually between 85 and 90 % and seldom fell below 80 %.¹

Instances of abnormally high proportions of non-protein-nitrogen in pasture herbage have, however, been observed, accompanied by digestive disturbances in stock grazing the herbage.² Thus, a sample of intensively manured grass at Castle Douglas [Watson, 1929] in May 1929 was found to contain 49 % of its total nitrogen in non-protein form, whilst in another at Retford [Greenhill, 1930, 2] in April 1930 the value was 38 %. Samples of intensive pasture herbage from a farm at East Clandon [Greenhill, 1930, 3] in March and April 1930 were also found to contain between 30 and 40 % of their nitrogen in non-protein form; although in this instance the herbage was not being grazed at the time, the possibility of its causing digestive disturbances in stock was suspected.

Although instances of serious digestive disturbances in stock grazing grass, due apparently to an abnormally high proportion of non-protein-nitrogen or of a particular form of non-protein-nitrogen in the herbage, are of rare occurrence, instances of minor disturbances attributed, rightly or wrongly, to such an excess are less uncommon. It was decided, therefore, in 1930 to investigate further the amounts and relative proportions of the nitrogen compounds occurring in grass.

EXPERIMENTAL.

As practically no data were available in the literature, concerning the amounts of the various nitrogenous constituents occurring in grass, it was decided to determine the amounts of some of these constituents present normally in intensive pasture herbage at different times of the season. Accordingly, a number of samples were examined in 1930.

¹ The occasional lower values obtained were traceable usually to some hydrolysis of protein having occurred between the times of taking and drying the sample.

² The so-called "grass tetany" of stock is often ascribed to an excess of non-protein-nitrogen or of a particular non-protein nitrogenous constituent in the herbage.

Particulars of samples examined.

Twelve samples at the 4–6 in. growth stage, obtained from the intensively manured rotational grazing paddocks in Leak's Meadow and Feeding Ground at Jealott's Hill, were examined in the months of May, August, September and October. The herbage examined was not necessarily representative of the whole paddock; in some cases this was so, whilst in others the sample was taken from those areas where growth was most luscious. Also, although the samples were all of herbage at the grazing stage, the pasture was not necessarily grazed at the time the sample was taken. In those cases where it was grazed, however, no digestive disturbances in the stock were observed.

Methods of examination employed.

Determinations of the total nitrogen and of the protein-, amide-, ammonia- and nitrate-nitrogen were made on the dried, ground material.

The total nitrogen was determined by the Kjeldahl and the protein-nitrogen by the Stützer method.

The amide-nitrogen was determined by boiling with 5 % HCl for 1½ hours and subsequent distillation with magnesia, allowance being made for the nitrogen originally present in the ammoniacal form.

The ammonia-nitrogen was determined by direct distillation with magnesia.

The nitrate-nitrogen (including any nitrite-nitrogen if present) was determined by the iron reduction method described by Vickery and Pucher [1929].

It should be noted [Greenhill, 1933] that grass kept for any appreciable length of time in the fresh condition is very liable, unless special precautions are taken, to undergo significant change in composition: besides some loss of dry matter, hydrolysis of protein, with a marked diminution in the value obtained for the protein-nitrogen and a corresponding rise in the non-protein-nitrogen value, is particularly likely to occur. For this reason it is important that as little time as possible should elapse between the taking and drying of the sample, and that the drying should be carried out as rapidly as possible, the grass being spread out in a thin layer and dried in an oven at a temperature of between 70° and 100°.

RESULTS.

The analytical data are summarised in the top section of Table I. The non-protein-N values, expressed as percentages of the total N, ranged from 12 to 23, the amide-N from 2.7 to 8.1, the ammonia-N from 1.0 to 2.0 and the nitrate-N from 0.1 to 4.0.

Comparing the individual samples, the proportions of the nitrogen present in amide and ammoniacal forms showed no relationship to the total nitrogen content of the herbage or to one another: the higher proportions of non-protein- and of nitrate-nitrogen, however, with exceptions, were associated with the higher total nitrogen contents.

DISCUSSION OF RESULTS AND CONCLUSIONS.

The samples of herbage examined included a wide range of total nitrogen contents (from the moderate figure of 2.8 to the high figure of 5.2 % of the dry matter, equivalent to 17.8 and 32.3 % of crude protein respectively) and also included samples taken under conditions of growth which might have been expected to be conducive to high proportions of non-protein-nitrogen. Also, the

Table I. *Summary of data for intensive pasture herbage (Jealott's Hill, Castle Douglas, Retford and East Clandon). 1929 and 1930.*

Particulars of samples	As % of dry matter		As % of total nitrogen				
	Total nitrogen	Equiv. to crude protein	Protein-N	Non-protein-N	Amide-N	Ammonia-N	Nitrate-N
12 samples intensive pasture herbage at grazing stage from Leak's Meadow or Feeding Ground, Jealott's Hill. May, August, September and October, 1930:							
Highest value	5.17	32.3	88	23	8.1	2.0	4.0
Lowest value	2.84	17.8	77	12	2.7	1.0	0.1
Average value	3.63	22.7	83	17	5.3	1.3	1.7
Intensive grass (first year permanent) from Castle Douglas [Watson, 1929]. May 1929. (Serious disturbance experienced in grazing stock.)	3.22	20.1	51	49	10.2	2.6	*
Intensive grass (seeds) from Retford [Greenhill, 1930, 2]. April 1930. (Serious disturbance experienced in grazing stock.)	5.21	32.6	62	38	11.3	4.1	2.5
4 samples intensive pasture herbage from East Clandon [Greenhill, 1930, 3]. March and April, 1930. (Pastures not being grazed at time as possibility of herbage causing some disturbance suspected.)	3.93	24.6	59	41	8.9	7.5	0.9
	3.80	23.8	67	33	5.2	2.9	0.6
	4.23	26.4	71	29	6.6	3.2	0.5
	4.42	27.6	68	32	6.3	2.2	0.3

* Present in appreciable amount.

paddock from which the Leak's Meadow samples were taken¹ had received heavier dressings of artificial fertilisers, including nitrogen, than are usually applied to pastures. In no instance however did the proportion of the total nitrogen present in non-protein form rise above 23 %, whilst in the majority of samples it did not rise above 16 %.

In the three lower sections of Table I are given the corresponding nitrogen values for the intensive herbage samples from Castle Douglas [Watson, 1929], Retford [Greenhill, 1930, 2] and East Clandon [Greenhill, 1930, 3] referred to earlier. Comparing the values for the Jealott's Hill samples with the values for these samples, it will be observed that whilst the total nitrogen contents of the herbage were comparable, the proportions of the nitrogen present in non-protein form were much higher in the latter than in the Jealott's Hill samples. Amide- and ammonia-nitrogen were present generally in higher proportions; the comparison shows, however, no definite difference in respect of the proportions of nitrate-nitrogen, which were very variable.

From a consideration of the data presented and referred to, it is concluded that the proportion of the total nitrogen present in protein form, in intensive herbage as grazed by stock, is normally more or less constant at a value between 80 and 90 %, and that only in exceptional instances does the non-protein-nitrogen exceed 20 % of the total nitrogen. Such instances may very occasionally occur, however, and may be liable to cause digestive disturbances in stock grazing the herbage (the seriousness of which will depend on the actual amount of non-protein-nitrogen or on the excess of some particular form of non-protein-nitrogen present, and on the general grazing and other conditions) and should be guarded against.

In general, it seems probable that an unusually high proportion of non-protein-nitrogen in grazing herbage is likely to arise only under a peculiar combination of circumstances, namely on heavily manured grass under conditions of very rapid growth or when, after heavy manuring followed by a check to growth,

¹ The Leak's Meadow samples were all taken from Paddock No. 1.

growth suddenly recommences. A high proportion of non-protein-nitrogen is associated nearly always, for these reasons, with a particularly leafy growth and, in consequence, with a high total nitrogen content, though, of course, leafy growth is not of itself to be considered in any way indicative of an unusually high proportion of non-protein-nitrogen. Further, from the nature of the causes, such instances are most likely to occur in the early and late weeks of the grazing season and to be of very transient occurrence.

Reference may be made to the data otherwise noted for the fractionation of the nitrogenous constituents of grass, and to further work on this question at this Station. Sjollem [1931], at the Veterinary Laboratory of the University of Utrecht, in an investigation of the cause of "grass tetany", examined, also in 1930, a large number of samples of intensive pasture herbage taken in the early and late months of the grazing season, with results generally confirming those obtained by the writer in this country. Chibnall and Miller [1931], examining a sample of perennial rye-grass from a sewage farm, found it to contain 12.7 % of its nitrogen in nitrate form, or calculated as KNO_3 , equal to 4.4 % of the dry matter; this grass, however, was of rank growth and not such as would be grazed, and further, the sample examined consisted of the upper leafy part of the herbage only. In America, Leukel and Coleman [1930] and Leukel *et al.* [1934], at the University of Florida Agricultural Experiment Station, have given data for the influence of different fertiliser and cutting treatments on the main nitrogenous constituents of several pasture grasses, whilst Archibald and Bennett [1932], at the Massachusetts Agricultural Experiment Station, studying the fractionation of the nitrogenous constituents in samples of herbage from manured and unmanured pastures and pure stands of white clover, found the ammonia-nitrogen to average about 1.8 % of the total nitrogen for the manured and about 1.0 % for the unmanured herbage; none of the samples contained more than slight traces of nitrate-nitrogen. At Jealott's Hill, Eggleton [1935] has studied the uptake of nitrogen by pasture herbage in the spring, following the application of various nitrogenous fertilisers, including sodium nitrite, and of sulphate of potash, while other investigations upon which work has been in progress since 1930 by Blackman and others, include the fractionation of the nitrogenous constituents in "early bite" pasture herbage and in lawn herbage, the results of which will be published later.

It is possible, at the present, to do little more than state the values for, and the general observations which have been made concerning, the fractionation of the nitrogenous constituents of grass, the factors probably determining the relative proportions of the different constituents having so far been but little studied. The influence of fertilisers, of climatic and seasonal conditions, and of soil conditions both chemical and biological, require study, whilst the fractionation needs to be applied throughout the period of growth and to different parts of the plant.

SUMMARY.

Following the very occasional occurrence of digestive disturbances in stock grazing intensively manured pastures, due apparently to an abnormally high proportion of non-protein-nitrogen or of a particular non-protein-nitrogen compound in the herbage, samples of intensively manured pasture at the grazing stage from the rotational grazing paddocks at Jealott's Hill were examined in the months of May, August, September and October 1930, for their contents of total, protein-, amide-, ammonia- and nitrate-nitrogen.

The total nitrogen contents of the herbage ranged from 2.8 to 5.2 % of the dry matter; the non-protein-nitrogen values, expressed as percentages of the

total nitrogen, ranged from 12 to 23, the amide-nitrogen from 2.7 to 8.1, the ammonia-nitrogen from 1.0 to 2.0, and the nitrate-nitrogen from 0.1 to 4.0. Comparing the individual samples, the proportions of the nitrogen present in amide and ammoniacal forms showed no definite relationship to the total nitrogen content of the herbage or to one another; the higher proportions of non-protein- and of nitrate-nitrogen, however, usually but not always were associated with the higher total nitrogen contents.

These values are compared with those of pasture samples associated with digestive disturbances in grazing stock at other centres, and the possible bearing of the relative proportions of the different forms of nitrogen compounds in grass in relation to their effect on the grazing of stock is discussed.

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LXVI. A NOTE ON THE POSSIBLE LIPOTROPIC ACTION OF ALKYLAMMONIUM COMPOUNDS.

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THE fact that choline, the nitrogenous constituent of lecithin, prevents the deposition of fat which occurs when rats are fed on a high fat diet, and also accelerates the removal of fat from fatty livers has been established by Best and his collaborators [1932; 1934; 1935]. This has been termed the "lipotropic action" of choline. It was found also that betaine, the acid corresponding to choline, is active in this respect but aminoethanol, the nitrogenous constituent of kephalin, is inactive. Both choline and betaine were administered as chlorides.

An attempt has been made to determine whether compounds related to choline chloride but without the hydroxyl group possess any lipotropic action. Trimethylammonium chloride (trimethylamine hydrochloride), trimethylethylammonium chloride, tetramethylammonium chloride and trimethylphenylammonium chloride have been used.

The following experiment was carried out to test the possibility that trimethylammonium chloride might influence liver fat. The rats were fed on a diet of mixed grain with added beef fat for 3 weeks, after which 15 were sacrificed to ensure that the livers had become fatty. The remainder were placed on a diet consisting entirely of sucrose. This procedure was shown by Best and Huntsman [1935] to cause a further accumulation of fat in the liver. One group of 15 animals received on the average 20 mg. of trimethylammonium chloride daily per rat (equivalent to about 100 mg. per kg.) and another, 20 mg. of choline chloride for comparison. A third group (controls) received sucrose only. The average liver fatty acid contents at the end of 12 days were as follows: trimethylammonium chloride 24.3 %, choline chloride 5.2 %, controls 16.7 %. Similar results were obtained when larger amounts of the amine, up to 100 mg. per rat per day, were given in other experiments.

The other substances tested, namely trimethylethyl-, tetramethyl- and trimethylphenyl-ammonium chlorides were prepared by the usual methods and analysed to determine purity. Such tetraalkylammonium compounds almost invariably exert a powerful pharmacological action of the curare type. As was expected, it was found that each of these compounds is extremely toxic even when administered orally in small doses in dilute solution. It was impossible to give a sufficient quantity to correspond with a known effective amount of choline chloride or to obtain evidence of lipotropic activity. In all cases where appreciable quantities were given the animals sickened, refused food and eventually died.

It is interesting to note that the triethyl homologue of choline (triethyl- β -hydroxyethylammonium hydroxide) has been shown by Channon and Smith [1936] to be effective in preventing deposition of liver fat although its action is not so intense as that of choline.

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LXVII. THE DETERMINATION OF LIGNIN IN THE ANALYSIS OF WOODS.

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Of all quantitative determinations in the analysis of plant materials the lignin determination is subject to greatest uncertainty since in the first place the constitution of this component is unknown and secondly all attempts to isolate it in the form in which it is believed to occur in the plant have, so far, failed. At the present time any so-called quantitative determination of lignin is at best a compromise, for the yield and composition of the product depend to a great extent on the analytical method employed. The best available methods for the routine determination of lignin in wood are those which involve the removal of the carbohydrates by hydrolysis with mineral acids. The details of the original methods and various modifications of them which have appeared from time to time are fully discussed by Schorger [1926, p. 518] and Hawley and Wise [1926, p. 163].

A number of modifications of the original methods [Ost and Wilkening, 1910; Willstätter and Zechmeister, 1913] have been suggested. Most of these aim at the preparation of a lignin complex, free from carbohydrate condensation products, and broadly speaking they involve either (1) preliminary treatment to remove those carbohydrates which are liable to form condensation products at some stage in the isolation of the lignin residue [Paloheimo, 1925; Ritter *et al.*, 1932; Norman and Jenkins, 1934], or (2) control of experimental conditions, such as the temperature of the wood samples during digestion with concentrated acid [Sherrard and Harris, 1932; Peterson *et al.*, 1932; Ritter *et al.*, 1932; Komarov, 1934]. Cohen and Dadswell [1931] have shown that with certain Australian woods the accurate determination of lignin is further complicated by the presence of gums or kinos which are resistant to concentrated mineral acids. These substances are, however, soluble in dilute sodium hydroxide, and the above authors have therefore proposed a modification of the Ost and Wilkening method which involves a preliminary treatment with this reagent. A further modification has since been suggested by Cohen [1934].

Since the crux of the lignin determination lies in the efficient removal of carbohydrates, it is essential that uniformity of method should be established for the digestion with concentrated acid and the subsequent hydrolysis with dilute acid. Although the method of Willstätter and Zechmeister [1913], which prescribes the use of 42 % hydrochloric acid, has hitherto been considered to yield the most accurate results, considerations of convenience have led to the almost universal use of 72 % sulphuric acid, as originally recommended by Ost and Wilkening [1910]. Further, it has been indicated by Harris *et al.* [1934] that the sulphuric acid method is capable of yielding a lignin with maximum methoxyl content and free from carbohydrates. Billington *et al.* [1933], conclude that the modified sulphuric acid method of Ritter *et al.* [1932] yields results which are

almost as satisfactory as those obtained by the hydrochloric acid method. None the less, although the use of sulphuric acid is generally favoured, there is a complete absence of agreement concerning the significant steps in the lignin determination. For instance, although all the above workers use 2 g. samples of wood flour Peterson *et al.* digest with 30 ml. of 72 % H_2SO_4 at 4° for 18 hours, Sherrard and Harris recommend 20 ml. of 70 % H_2SO_4 at 10° for 16 hours, Ritter *et al.* recommend 25 ml. of 72 % H_2SO_4 at 20° for 2 hours and Komarov digests his wood samples for $2\frac{1}{2}$ hours at $20\text{--}21^\circ$ in the case of softwoods and $25\text{--}26^\circ$ in the case of hardwoods.

The method which has been in current use in this laboratory is substantially that recommended by Schorger [1926, p. 524] except that 12.5 ml. of 72 % sulphuric acid have been used per 2 g. (air-dry) sample of wood, and the hydrolysis with 3 % acid has been carried out under a reflux condenser. The following work was undertaken with a view to revising this method, and it has necessarily involved the assessment of the relative merits of several of the methods referred to above. For the present, the question of the eradication of gums and kinos has been left aside.

EXPERIMENTAL.

The effect of a preliminary hydrolysis with dilute sulphuric acid on the yield of apparent lignin.

Norman and Jenkins [1933] have shown that in the case of certain straws the yield of apparent lignin can be substantially reduced by a pre-treatment with 5 % sulphuric acid. They also record small losses in the apparent lignin of Scots pine and beech wood due to the same treatment. However, as mentioned in the Forest Products Research Board Report [1933], experiments carried out in this laboratory indicate that this expedient is not applicable to all woods. The experimental data in Tables I and II support this view. In these and succeeding tables results are expressed as percentages by weight of oven-dry original 80–100 mesh wood.

Table I. *The effect of preliminary hydrolysis with 1% sulphuric acid for 5 hours at 100° on the apparent lignin content of wood.*

16 hours' digestion with 12.5 ml. 72% H_2SO_4 at $15\text{--}20^\circ$; 2 hours' hydrolysis with 3% acid.

Material	Apparent lignin content		Change in apparent lignin content
	Untreated sample	After preliminary hydrolysis with 1% H_2SO_4	
African pencil cedar (<i>Juniperus procera</i> Hochst.)	37.10	40.77	+ 3.67
Louisiana Gulf cypress (<i>Taxodium distichum</i> Rich.):			
Heartwood (50 mesh)	31.82	32.08	+ 0.26
Sapwood	34.44	34.77	+ 0.33
Pitch pine (<i>Pinus palustris</i> Mill.):			
Highly resinous	21.18	21.43	+ 0.25
Slightly resinous	29.60	29.89	+ 0.29
English oak (<i>Quercus</i> sp.)	22.40	18.97	– 3.43
Jarrah (<i>Eucalyptus marginatus</i> Sm.)	43.37	39.97	– 3.40
Teak (<i>Tectona grandis</i> L.f.)	30.53	31.76	+ 1.23
Abura (<i>Mitragyna stipulosa</i> Kuntze)	32.75	33.40	+ 0.65

In all cases, except oak and jarrah (Table I), increases in apparent lignin are observed. These increments probably arise from the formation of insoluble products from partially hydrolysed carbohydrates, although in the light of more recent knowledge, it is uncertain whether the cause of the increments is to be sought in the conditions of the preliminary hydrolysis alone.

Preliminary hydrolysis with 5% sulphuric acid.

Three of the woods used in Table I were selected for the determinations recorded in Table II, the preliminary hydrolysis being carried out on 2 g. (air-dry) samples of wood flour with 100 ml. 5 % sulphuric acid at 100° for 3 hours. The wood residue was in each case collected in an alundum crucible (porosity RA 98) in the usual manner.

Table II. *The effect of preliminary hydrolysis with 5% sulphuric acid for 3 hours at 100° on the apparent lignin content of wood.*

Material	Apparent lignin content		
	Untreated sample	After hydrolysis with 5% H_2SO_4 for 3 hours at 100°	Change in apparent lignin content
African pencil cedar	37.59	39.18	+ 1.59
Louisiana Gulf cypress	32.67	32.52	0.15
English oak: sapwood	23.10	20.31	- 2.79

The only results that can be directly compared with those of Norman and Jenkins [1934] are those for oak and teak. For oak wood, these authors observed a loss of 8.56 parts of apparent lignin and for teak a loss of 2.03 parts, after hydrolysing for 1 hour with 5 % sulphuric acid at 100°. Since, however, of the 7 species examined here only two show a decrease in apparent lignin, it is obvious that the introduction of this type of preliminary hydrolysis as a refinement in the lignin determination is open to criticism.

Preliminary hydrolysis with dilute sulphuric acid with immediate removal of the products of hydrolysis.

A more successful pre-treatment was suggested by the work of Paloheimo [1929] who carried out two successive digestions with 70 % sulphuric acid. The concentration of hydrolysis products during the second digestion was thus considerably reduced, and for rye straw a lower yield of lignin was obtained.

In the following series of experiments (Table III) 2 g. samples of air-dry wood in alundum crucibles of porosity RA 98 were hydrolysed either before or after extraction with alcohol-benzene with boiling dilute sulphuric acid in a series of stages. 25 ml. of acid were added at each stage and removed by suction along with the hydrolysis products. After the final stage the samples were washed free from acid with hot water before carrying out the usual lignin determination.

These results show that it is possible by using 5 % acid to reduce the apparent lignin content of beech and African pencil cedar by over 1 % without appreciable loss of methoxyl. 10 % sulphuric acid appears to attack the lignin of beech wood since the methoxyl is depleted.

Table IV shows the distribution of pentosans after treatment (3). The loss of 1 % apparent lignin in the case of cedar is apparently not due to the removal of pentosans. On boiling the acid extract for 2 hours, however, an insoluble residue resembling lignin was obtained. This amounted to 0.35 % in

Table III. *The effect of a preliminary hydrolysis with removal of hydrolysis products on the apparent lignin contents of beech and African pencil cedar.*

Treatment	African pencil cedar			Beech		
	Lignin	Methoxyl in lignin (i)	(i) as % of lignin	Lignin	Methoxyl in lignin (ii)	(ii) as % of lignin
1. Original wood	37.73	4.95	13.12	23.53	4.79	20.35
2. Alcohol-benzene extraction followed by treatment with 250 ml. hot 1% H_2SO_4 for 2-min. periods	37.96	5.00	13.18	24.49*	4.71	19.25
3. Alcohol-benzene extraction followed by treatment with 250 ml. hot 5% H_2SO_4 for 2-min. periods	36.72	4.91	13.36	22.00	4.52	20.55
4. Alcohol-benzene extraction followed by treatment with 500 ml. hot 5% H_2SO_4 for 2-min. periods	37.56†	5.03	13.41	23.36†	4.50	19.27
5. Treatment with 250 ml. hot 5% H_2SO_4 for 2-min. periods prior to alcohol-benzene extraction	36.63	4.78	13.05	22.71	4.59	20.22
6. Treatment with 250 ml. hot 5% H_2SO_4 for 4-min. periods prior to alcohol-benzene extraction	36.54	4.77	13.06	22.45	4.63	20.63
7. Treatment with 250 ml. hot 10% H_2SO_4 for 2-min. periods prior to alcohol-benzene extraction	37.19	4.93	13.25	22.02	4.26	19.83

* Temperature of digestion with 72% H_2SO_4 , 25.5–29°.† Temperature of digestion with 72% H_2SO_4 , 24.5°.Table IV. *Distribution of pentosans after treatment.*

	African pencil cedar	Beech
Total pentosans in original wood	11.10	25.59
Pentosans in residual wood after treatment (3)	10.85	23.47
Pentosans in 5% H_2SO_4 extract	Nil	1.12
Insoluble residue from 5% H_2SO_4 extract	0.35	0.11
5% sulphuric acid extract	6.55	2.56

the case of cedar, and it would undoubtedly have been estimated as lignin had not precautions been taken to remove the products of hydrolysis as they were formed (*cf.* Table II).

Unfortunately in the experiments in which 500 ml. of 5% sulphuric acid were used the digestions with 72% acid were carried out at a room temperature of 24.5°, some 5–8° higher than in the other experiments. This made the results useless for their original purpose, since the unexpectedly high values obtained for apparent lignin were in all probability due to the effect of increased temperature. Before continuing with the investigation of hydrolytic pre-treatments therefore a series of determinations was carried out to determine the maximum increase in apparent lignin that might be caused by summer temperatures in this country.

The influence of summer temperatures on the lignin determination.

Lignin determinations were made on a number of woods according to the method which has been in current use in this laboratory, except that the digestion with 72 % sulphuric acid was carried out in an oven maintained at 33°, the highest laboratory temperature likely to occur during summer in England. Table V shows these results compared with those obtained with the same woods at temperatures of 15–20°.

Table V. *Effect of temperature of digestion with 72% sulphuric acid on apparent lignin content.*

Temperature during digestion with 72% H ₂ SO ₄	15–20°			33°			Increase in apparent lignin due to temperature
	Apparent lignin	Methoxyl in lignin (i)	(i) as % of lignin	Apparent lignin	Methoxyl in lignin (ii)	(ii) as % of lignin	
African pencil cedar	37.73	4.95	13.12	40.00	4.98	12.44	2.27
Louisiana Gulf cypress	32.67	5.21	15.95	35.20	5.20	14.77	2.53
Sitka spruce (<i>Picea sitchensis</i> Carr.)	27.83	4.14	14.88	29.74	4.23	14.23	1.91
Pitch pine, slightly resinous	29.19	4.27	14.63	31.15	3.70	11.89	1.96
Norway spruce (<i>Picea Abies</i> Karst)	26.05	4.10	15.73	27.95	4.04	14.45	1.90
Beech (<i>Fagus sylvatica</i> Linn.)	23.53	4.79	20.35	27.98	4.95	17.70	4.45
Ash (<i>Fraxinus excelsior</i> Linn.)	24.75	4.92	19.88	28.88	5.05	17.48	4.13
English oak	22.40	3.90	17.44	27.43	3.86	14.03	5.03
Jarrah	13.37	5.84	13.48	47.94	5.99	12.48	4.57

It can be seen that a relatively high temperature affects the yield of hardwood lignin much more than that of softwood lignin. This may be accounted for by the greater proportion of readily hydrolysable carbohydrates present in hardwoods. These results amply confirm the observations of Sherrard and Harris [1932] and Peterson *et al.* [1932].

The optimum conditions for carrying out the determination of lignin with 72% sulphuric acid.

Ritter *et al.* [1932] have published graphs showing the effect of temperature on the yield of lignin from the wood of sugar maple after digestion with 72 % sulphuric acid for periods ranging from 1 to 96 hours. They conclude that a digestion for 2 hours at 20° ensures complete solution of the carbohydrates with a minimum amount of decomposition and adopt this for future use, although it would appear from their graphs that a minimum yield of lignin is actually obtained by digesting for 7 hours at 10°. Consideration of these graphs and the results of other workers [Sherrard and Harris, 1932; Peterson *et al.*, 1932] has led us to adopt 10° as the best temperature for the digestion.

The authors desire to record here their indebtedness to Mr J. Piqué of the Low Temperature Research Station, Cambridge, for supplying the design of a constant temperature chamber in which it has been possible to maintain a

temperature of $10 \pm 0.5^\circ$ for prolonged periods. Full details of the complete apparatus can be obtained on request from the Director of Forest Products Research.

The effect of crucible porosity on the yield of apparent lignin from certain hardwoods.

A set of determinations was carried out on beech, ash and English oak to compare the efficiency of alundum crucibles of porosity RA 98 and 360 respectively in retaining the lignin precipitate (Table VI).

Table VI. *The effect of crucible porosity—RA 98 compared with RA 360.*

16 hours' digestion with 72% H_2SO_4 at 10° ; 2 hours' hydrolysis with 3% acid.

Volume of 72% H_2SO_4 used ml.	Beech				Oak, sapwood				Ash			
	Apparent lignin		Methoxyl in lignin		Apparent lignin		Methoxyl in lignin		Apparent lignin		Methoxyl in lignin	
	RA 98	360	98	360	98	360	98	360	98	360	98	360
12.5	22.36	22.81	4.61	4.63	19.92	20.21	4.17	4.18	23.24	23.57	4.75	4.78
20	22.25	22.60	4.65	4.69	19.34	19.54	4.13	4.16	22.54	23.17	4.69	4.72
25	22.28	22.30	4.58	4.67	19.10	19.25	4.02	4.13	22.43	23.00	4.70	4.78
30	21.82	22.10	4.48	4.63	18.86	19.44	4.00	4.13	22.27	22.87	4.67	4.77

In all cases a slightly higher yield of apparent lignin was obtained by using crucibles of porosity RA 360 and the methoxyl content of the lignin collected in these was constant irrespective of the volume of sulphuric acid used. Crucibles of porosity RA 360 have therefore been adopted for collecting lignin in all future work.

The influence of the volume of 72% sulphuric acid used on the yield of apparent lignin.

2 g. samples of air-dry, alcohol-benzene (1:2)-extracted sawdust were digested with different volumes of 72% sulphuric acid at 10° for 16 hours. The acid was then diluted to a concentration of 3% and the mixture boiled for 2 hours before collecting the lignin residue (Table VII).

Table VII. *The effect of using different volumes of 72% sulphuric acid.*

Volume of 72% H_2SO_4 used ml.	Beech		African pencil cedar		English oak sapwood		Norway spruce		Ash		Douglas fir	
	Meth-		Meth-		Meth-		Meth-		Meth-		Meth-	
	Appar-	oxyl	Appar-	oxyl	Appar-	oxyl	Appar-	oxyl	Appar-	oxyl	Appar-	oxyl
	ent in	in	ent in	in	ent in	in	ent in	in	ent in	in	ent in	in
	lignin	lignin	lignin	lignin	lignin	lignin	lignin	lignin	lignin	lignin	lignin	lignin
12.5	22.81	4.63	38.06	4.94	20.21	4.18	25.63	4.15	23.57	4.78	26.80	4.02
20	22.60	4.69	37.81	5.07	19.54	4.16	25.37	4.09	23.17	4.72	26.55	3.92
25	22.30	4.67	37.33	4.95	19.25	4.13	25.42	4.07	23.00	4.78	26.54	3.99
30	22.10	4.63	37.45	4.94	19.44	4.13	25.33	4.08	22.87	4.77	26.44	4.01

Consideration of the data suggests that for a 2 g. sample of wood containing approximately 10% of moisture 25 ml. of 72% sulphuric acid be adopted.

The effect of varying the time of contact of wood samples with 72% sulphuric acid on the yield of apparent lignin.

Table VIII gives the results obtained by digesting 2 g. samples of various woods with 25 ml. of 72% sulphuric acid at 10° for periods of 3 to 16 hours, the acid being subsequently diluted to 3% and the mixture boiled for 2 hours before collecting and washing the lignin in the usual way.

Table VIII. *The effect of varying the time of contact of wood with 25 ml. 72% sulphuric acid.*

Time of digestion with 72% H_2SO_4 at 10° (hours)	Beech		Cedar		English oak, sapwood		Norway spruce		Ash		Douglas fir	
	Meth-		Meth-		Meth-		Meth-		Meth-		Meth-	
	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin
3	34.58	4.70	42.83	5.58	25.55	4.07	30.88	4.56	30.96	4.81	34.05	4.23
4	21.60	4.46	42.02	5.44	19.00	3.94	25.58	4.14	23.12	4.69	30.43	4.31
5	21.60	4.45	37.31	4.90	18.47	3.92	25.34	4.05	22.50	4.69	26.77	3.96
6	21.62	4.49	37.27	4.99	19.03	4.05	25.14	4.04	22.75	4.65	26.61	3.87
7	21.70	4.47	37.23	5.01	18.86	4.05	25.40	4.06	22.67	4.76	26.70	3.82
16	22.30	4.67	37.33	4.95	19.25	4.13	25.42	4.07	23.00	4.78	26.54	3.99

The data suggest that for the woods examined hardwoods require 5 hours' digestion with 72 % sulphuric acid, whilst softwoods require 6 hours' digestion.

The effect of varying the time of hydrolysis with 3% sulphuric acid on the yield of apparent lignin.

The conditions of the final hydrolysis with dilute acid have perhaps been subject to fewer modifications than any other part of the lignin determination. Nevertheless Ritter *et al.* [1932] advocate an extension of the time of hydrolysis at the boiling-point of the acid from the usual 2 to 4 hours, whilst Komarov [1934] appears to consider that 1 hour's hydrolysis with acid of 5 % concentration is sufficient.

After extraction with alcohol-benzene (1:2) 2 g. samples of air-dry wood were digested with 25 ml. of 72 % sulphuric acid at 10° for 5 hours (Table IX(a)) and 6 hours (Table IX(b)). The acid was diluted to 3 % and the final hydrolysis at the boiling-point was carried out for various times.

Table IX. *The effect of varying time of hydrolysis with 3% sulphuric acid on the yield of apparent lignin, using:*

(a) 25 ml. 72% H_2SO_4 for 5 hours at 10°.

Time of hydrolysis with 3% acid (hours)	Beech		Cedar		English oak, sapwood		Norway spruce		Ash		Douglas fir	
	Meth-		Meth-		Meth-		Meth-		Meth-		Meth-	
	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin	Appar- ent lignin	oxyl in lignin
1	21.62	4.47	37.37	5.06	18.61	3.94	25.73	4.17	22.44	4.70	26.77	4.01
2	21.60	4.45	37.31	4.90	18.47	3.92	25.34	4.05	22.50	4.69	26.77	3.96
3	21.64	4.51	37.75	5.04	18.49	3.95	25.50	4.19	22.23	4.70	26.50	4.01
4	21.64	4.59	37.86	4.99	18.51	4.03	25.48	4.12	22.20	4.70	26.67	3.99

(b) 25 ml. 72% H_2SO_4 for 6 hours at 10°.

Time of hydrolysis with 3% acid (hours)	African pencil cedar		Norway spruce		Douglas fir	
	Apparent lignin	Methoxyl in lignin	Apparent lignin	Methoxyl in lignin	Apparent lignin	Methoxyl in lignin
1	37.89	4.99	25.36	4.07	26.83	3.92
2	37.27	4.99	25.14	4.04	26.61	3.87
3	37.53	4.98	25.14	4.11	26.65	3.94
4	37.49	4.93	25.36	4.17	26.65	3.98

These results indicate that with the exception of ash wood 4 hours' hydrolysis with 3 % sulphuric acid is unnecessary for the woods examined. Moreover, in particular cases, the longer hydrolysis may lead to high values for lignin. From a practical standpoint it is obviously important that the time taken for the complete lignin estimation should be reduced to a minimum and therefore 2 hours is proposed as the time for the hydrolysis with 3 % sulphuric acid.

The action of concentrated sulphuric acid on sugars as a source of error in the lignin determination.

Hilpert and Littmann [1934] have confirmed and amplified the earlier work of Paloheimo [1929] on the action of concentrated mineral acids on sugars in relation to the determination of lignin and have shown that whereas glucose yields 0.81 % of an insoluble lignin-like product when treated with 72 % sulphuric acid for 48 hours at 20–22°, fructose may yield 25 %, sucrose 13–14 % and xylose 36 %. This so-called resinification of sugars is regarded as an important source of error in the lignin determination in plant materials generally, but in a later paper [1935] it is shown that the reaction is profoundly affected by changes in temperature. At 6° fructose and xylose only yield 0.26 and 0.01 % respectively of an insoluble lignin-like residue.

In the following experiments 1 g. samples of pure xylose, fructose and sucrose respectively were mixed with 25 ml. 72 % sulphuric acid at 10° and maintained at that temperature for 5 hours. Any insoluble precipitate formed was collected in an alundum crucible of porosity RA 360, washed with water and dried at 105° (Table X).

Table X. *Effect of 72% sulphuric acid on sugars at 10°.*

	Insoluble residue (%)
Xylose	0.07
Sucrose	0.04
Fructose	0.01

It may be concluded that the possible error introduced into the determination of lignin in wood by the presence therein of small quantities of sugars can hardly be greater than the experimental error of the determination, provided that the digestion with 72 % H_2SO_4 is carried out at 10°.

DISCUSSION.

The experimental work described above will have served a useful purpose if it only succeeds in focussing attention on the wide variations in technique at present being employed in different laboratories in the estimation of lignin by means of sulphuric acid. From a consideration of all the available data on this subject, there can be no doubt that so long as the majority of workers continue to estimate lignin by the same general method the conditions of the digestion with concentrated acid must be standardised. Reasons have been given for the adoption of 10° as the most suitable temperature for this digestion and of 25 ml. as the most suitable volume of 72 % sulphuric acid. The question of the most appropriate time of digestion may not be so easily settled, but the data show that it can be shorter for hardwoods than for softwoods.

The preparation of the wood sample for the main hydrolysis is the step in the determination which will from time to time be subject to the greatest

modifications. Chemically the greatest variants in wood substance are the minor components and it is not surprising that as more species of wood are examined it is being found that the minor components cannot always be effectively removed by the usual neutral solvents. This has become particularly apparent in certain of the Australian hardwoods examined by Cohen and his co-workers [1931; 1934].

It is highly probable that the removal of minor components prior to the lignin determination proper will ultimately constitute a different problem for different types of wood, but further discussion of this aspect of the problem would appear to be unprofitable at present. Meantime thorough extraction with alcohol-benzene (1:2) followed by washing with hot water is recommended for woods which are not conspicuously high in extractives in the belief that further work on the extractive problem can best be interpreted after the main steps in the lignin determination proper have been settled.

Greater accuracy may conceivably be introduced into the lignin determination by applying a preliminary hydrolysis with dilute acid, provided that the products of hydrolysis are removed as soon as possible. In this connection the results obtained in the earlier part of this investigation are encouraging and the matter will be followed up as time permits.

SUMMARY.

1. Current methods for determining lignin in wood by means of 72% sulphuric acid are briefly reviewed and the present lack of uniformity in these methods is stressed.

2. Preliminary hydrolysis of the wood sample with dilute sulphuric acid has been shown to be ineffective as a general means of preventing the formation of carbohydrate condensation products during the isolation of lignin, unless precautions are taken to remove the products of hydrolysis as soon as possible after they are formed.

3. The conditions governing the digestion of the wood sample with 72% sulphuric acid have been studied in detail and on the basis of the data obtained the following procedure is suggested for the determination of lignin in wood. After a preliminary extraction with alcohol-benzene (1:2), a 2 g. sample of air-dry wood of approximately 10% moisture content is digested with 25 ml. of 72% sulphuric acid at $10^{\circ} \pm 0.5^{\circ}$ for 5 hours in the case of hardwoods and 6 hours in the case of softwoods. The acid is diluted with water to a concentration of 3% and the mixture boiled under reflux for 2 hours. The lignin residue is then collected in an alundum crucible of porosity RA 360, washed till free from acid and dried at 105° .

4. It has been shown that when xylose, fructose and sucrose are treated with sulphuric acid under the conditions stated above only negligible amounts of insoluble residues are obtained.

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LXVIII. THE DESTRUCTION OF 1:2:5:6-DIBENZANTHRACENE IN THE MOUSE.

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1:2:5:6-DIBENZANTHRACENE, injected into the breast muscles of a fowl, disappears from the site of injection in a few weeks [Chalmers, 1934; Berenblum and Kendal, 1934]. It seemed important to determine whether disappearance of dibenzanthracene was due to destruction, transportation to another part of the body or excretion unchanged. Preliminary evidence of destruction or excretion was forthcoming from an unsuccessful attempt to induce tumours of the reticulo-endothelial system of fowls: 20 fowls, which were given 30 weekly intravenous injections amounting to 20 mg. total dibenzanthracene (as colloidal aqueous solution), developed no tumours. Neither did they contain any dibenzanthracene in eight typical tissues when these were examined 2 years after commencement. Yet control experiments showed that dibenzanthracene was distributed at least to the heart, lungs and liver directly after intravenous injection (8 mg.).

Of course, proof of destruction of dibenzanthracene in the body actually requires an examination of the whole animal and its excreta for dibenzanthracene. Therefore, using mice as experimental animals, two methods were devised for quantitatively extracting the whole animal, obtaining an extract as free as possible from body pigments or fluorescent impurities and estimating dibenzanthracene spectrographically. The rate of disappearance of dibenzanthracene was then studied by estimating the amounts persisting in different mice at varying intervals after a standard intraperitoneal injection, while the question of excretion was studied by extracting the total excreta and examining such extracts for any traces of dibenzanthracene.

Experimental.

Preparation of extracts for spectrophotography (Method 1). The dead mouse was heated on a water-bath with 20 ml. 40% NaOH for a few hours. The sediment was separated and disintegrated with a little conc. HCl and the whole then neutralised with HCl and made alkaline with 5–10 g. anhydrous Na₂CO₃ (to prevent subsequent extraction of coloured material into the benzene). The mixture was evaporated to dryness on a water-bath and finally desiccated in a hot-air oven at 110° for several hours. The solid was finely powdered, excess of anhydrous Na₂SO₄ added and the whole extracted with boiling benzene either in a Soxhlet apparatus or by several changes of benzene under a reflux condenser. The extract was finally made up to a known volume (usually 100 ml.).

Numerous control tests with known quantities of dibenzanthracene gave reasonably consistent results. The amount of coloured impurities was very low, though it tended to vary somewhat. The chief objection to the method is the amount of trouble and time necessary to carry out a single extraction.

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Method 2. This is a simplified method which was used in later work (Table I) with a similar accuracy and efficiency. The mouse was boiled under a reflux condenser with alcoholic KOH (10 ml. saturated aqueous KOH and 80 ml. alcohol). 100 ml. benzene and 10–15 g. anhydrous CaCl_2 were added after cooling and the mixture was boiled for a further 10–15 min., then cooled and 8–9 ml. conc. HCl added. Most of the supernatant fluid was poured into a large separating funnel and shaken with several large quantities of dil. HCl to wash out all the alcohol (the acid being necessary to prevent the dibenzanthracene from passing into the watery phase as a colloidal solution). After a final washing with water it was shaken with several lots of dil. Na_2CO_3 solution (for the removal of pigments and other impurities) and filtered. A clear, almost colourless solution was obtained, representing all the dibenzanthracene from the mouse.

Estimation of the dibenzanthracene content of the extracts. The quantitative estimations of dibenzanthracene in mouse extracts were made by comparing the intensities of the characteristic fluorescent bands with those of a series of standard solutions of dibenzanthracene, photographed on the same plate. The apparatus employed was identical with that used by Hieger [1930] and others for the detection of carcinogenic compounds. The experimental error was less than 20 % while the limit of sensitivity was about one part of dibenzanthracene in 5–10 million of benzene, representing 0.01–0.02 mg. per mouse.

The disappearance of dibenzanthracene from the mouse. 2 mg. 1:2:5:6-dibenzanthracene dissolved in 0.5 ml. lard (previously filtered at 37°) were injected intraperitoneally into each of 44 mice. Two of the mice were killed and examined immediately and the remainder at intervals, after killing or death through natural causes, over a period of 160 days. A second group of 40 mice received intraperitoneal injections of a colloidal solution of dibenzanthracene in water. This solution contained about 16 mg./100 ml. and was injected in 6 doses of 2 ml. spread over a period of 4 days (representing about 1.9 mg. per mouse). Three mice were killed after the last injection and were found to contain 1.8 mg. each. Further estimations were carried out at intervals as in the previous group.

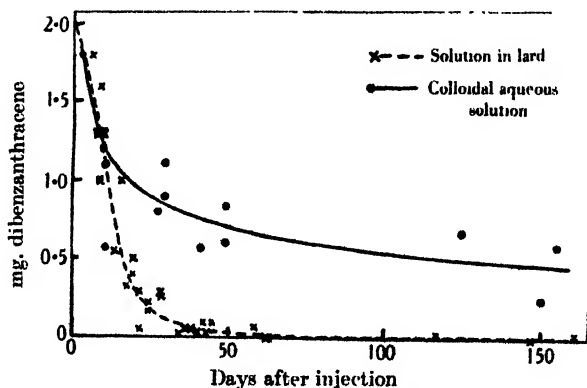


Fig. 1. 1:2:5:6-Dibenzanthracene detected in mice after intraperitoneal injection.

The results show clearly (Fig. 1) that dibenzanthracene disappeared from both groups of mice during the first 3 weeks. The disappearance was almost complete with the former group, whereas only about two-thirds disappeared with the latter. The following additional conclusions were reached from naked-eye and histological examinations of the animals: (1) Following the

injections of colloidal dibenzanthracene, small white nodules were found in the peritoneal cavity, usually attached to the viscera. These were present even at the end of the experiment and were found to be inflammatory nodules containing varying amounts of dibenzanthracene. Two such nodules in mice killed after 200 days contained 0.14 and 0.25 mg. of dibenzanthracene respectively. (2) In the group injected with the lard solution 1 of 9 mice surviving 20 weeks developed a tumour; in the group injected with colloidal solution in water, 4 of 9 developed tumours. These were all spindle-cell sarcomata arising in the peritoneal cavity and invading the surrounding tissues.

Other short experiments, with minor modifications in procedure, were carried out to confirm the above results. 12 mice received an intraperitoneal injection of 1 ml. of lard containing 1 mg. of dibenzanthracene (group A) and further 12 mice were given three daily intraperitoneal injections of colloidal dibenzanthracene, amounting to 1.2 mg. per mouse (group B). 5 mice from each group were killed immediately after injection (*i.e.* on the first day in group A and on the third day in group B) and the remainder were killed 12 days later. The mice were extracted (by method 2 described above) and the dibenzanthracene content was estimated in each.

Table 1. *Dibenzanthracene detected in mice after intraperitoneal injections.*

Directly after injection		After 12 days	
Lard solution mg.	Colloidal solution mg.	Lard solution mg.	Colloidal solution mg.
1.05	1.2	0.39	0.46
1.0	1.1	0.44	0.33
0.95	0.8	0.25	0.72
1.0	0.9	0.44	0.12
0.9	1.0	0.33	0.50
		0.46	0.50
		0.52	0.40
Average 0.98	1.0	0.40	0.43

The results (Table I) confirm those of the previous experiment. Part of the dibenzanthracene injected had disappeared, the amounts being about the same in both groups, as was the case in the previous experiment on the 12th day.

The possibility of excretion of unchanged dibenzanthracene. Intraperitoneal injection of 1 ml. of lard containing 3.6 mg. dibenzanthracene was made into each of 5 mice. These mice were kept isolated in separate glass jars with a thick layer of loose asbestos fibre at the bottom of each jar. This, together with the accumulated excreta and food debris, was dried and extracted with benzene after 14 days, when the mice were killed and also extracted. In the 5 mice, 1.10, 1.00, 0.90, 1.25 and 0.95 mg. dibenzanthracene respectively were found, averaging 1.04 mg. per mouse. Thus about 2.6 mg. per mouse had disappeared. Yet no dibenzanthracene could be detected in the extracts of the excreta. Since the latter were rather coloured, the following control test was performed. Extracts of 14-day excreta from control mice were examined with known additions of dibenzanthracene. Detection of the compound in these mixtures was possible with quantities as small as 0.04 mg. Since therefore less than 0.04 mg. of the missing 2.6 mg. dibenzanthracene was present in the excreta in an unchanged form, dibenzanthracene must be metabolised in the body into a form no longer possessing the characteristic fluorescent bands.

DISCUSSION.

The power of the animal body to metabolise dibenzanthracene is of some interest. Dibenzanthracene is a stable aromatic hydrocarbon, insoluble in water and only sparingly soluble in a few organic solvents, whilst there is no existing evidence of enzymes in the body which might catalyse its destruction. However, the recent work on the relation between cholesterol, bile acids and oestrogenic compounds would suggest that the body is capable of more complex chemical reactions than have previously been considered.

The chief interest of the present findings lies in the fact that 1:2:5:6-dibenzanthracene is one of a group of related carcinogenic compounds. Its somewhat rapid destruction in the body therefore raises the question whether carcinogenic action is due to dibenzanthracene itself or to products of its decomposition. In the above experiments, more tumours were developed when an appreciable amount of dibenzanthracene persisted in the body for many months (*i.e.* following injections of the colloidal solution) than when only traces of the compound remained after the first 3 weeks (following injections in lard). This suggests that dibenzanthracene is the carcinogenic agent but no conclusion based on such slight evidence merits any emphasis. The persistence of dibenzanthracene in the body when injected in colloidal solution is probably due to aggregation into nodules in the peritoneal cavity with consequent encapsulation.

It has been suggested that carcinogenic compounds appear in the body through some disturbance in sterol metabolism [Kennaway and Cook, 1932]. If however these compounds are destroyed in the body as readily as dibenzanthracene, they will have to appear with corresponding rapidity, if experimental evidence for this suggestion is ever to be obtained.

SUMMARY.

1. A method for the quantitative estimation of 1:2:5:6-dibenzanthracene in the mouse is described.
2. Estimation of dibenzanthracene at different times after intraperitoneal injection (2 mg.) showed that the greater part disappeared from the animal. The disappearance was more complete when the dibenzanthracene was injected dissolved in lard than when it was injected in the form of a colloidal solution in water. No dibenzanthracene could be detected in the excreta of such animals.
3. It is therefore concluded that the mouse is able to metabolise dibenzanthracene.

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LXIX. FRUCTOSE METABOLISM IN THE INTACT ANIMAL.

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THE work described in this paper was undertaken to obtain fresh evidence on the metabolism of fructose in the intact rabbit. Many of the earlier workers, handicapped by the lack of a reliable method for estimating fructose in blood, estimated only the total sugar present or confined their attention to the amount of glycogen deposited in the liver and muscles after administration of fructose. With the development of new and reliable methods for the estimation of fructose in comparatively small quantities of blood it has become possible to attack the problem from the point of view of the blood chemistry, and this method has been employed in obtaining the results which follow.

MacLean and Smedley [1912] showed that the hearts of the dog and rabbit were unable to utilise fructose and this result was confirmed by Steinberg [1927] and by Stewart and Gaddie [1934] for frogs' hearts, while Ashford [1933] obtained similar negative results using brain tissue. Dickens and Greville [1932] found that some embryonic tissues were able to convert only glucose into lactic acid, whilst others were able to convert fructose as well. The intact animal is capable of utilising fructose but there are clearly two possibilities (1) that fructose must first be converted into glucose or into glycogen in the liver, and (2) that some of the tissues are able to utilise it directly.

Cori and Cori [1928] have concluded that fructose is utilised directly by the tissues without previous conversion into glucose, whilst Mann and Bollmann [1930], working with hepatectomised animals, support the view that fructose is first converted into glucose. Mann [1925, 1, 2] showed that glucose but not fructose could restore a moribund hepatectomised dog and further evidence of the importance of the liver in the disposal of fructose has been provided by Isaac [1920], Stroebe [1932] and Kimball [1932], who showed that fructose causes prolonged hyperglycaemia in cases of hepatic disease but not in normal animals, while Bodansky [1923] and Kimball [1932] have demonstrated the importance of fructose tolerance tests in indicating liver damage.

EXPERIMENTAL.

Estimation of fructose.

The estimation of fructose in presence of glucose in small amounts of blood involves certain difficulties. In the early experiments in this series fructose was estimated by a method depending on the reduction of phosphomolybdotungstic

¹ In receipt of a part time grant from the Medical Research Council.

acid. This method had certain advantages over the colorimetric method of Van Creveld [1927] and Radt [1928] but it suffered from the disadvantage that the reaction was incomplete and that any attempt to force it to completion caused interference by glucose. This method however, when the conditions were carefully controlled, gave satisfactory results which were found to be comparable with those obtained by Van Creveld's method.

Later a micro-scale modification of Patterson's [1935] adaptation of Radt's method was evolved which was more convenient and gave results similar to those obtained by the previous method. The results which follow were obtained in most cases by employing both methods.

1. *Phosphomolybdotungstic acid method.* To 2.0 ml. of Folin-Wu blood filtrate 2.0 ml. of the phosphomolybdotungstic acid reagent (35 g. molybdic acid and 5 g. sodium tungstate were dissolved in 200 ml. of 10 % NaOH and the mixture boiled vigorously, cooled, diluted to 350 ml., added to 125 ml. 85 % H_3PO_4 and diluted to 500 ml.) were added and the mixture heated on the boiling water-bath for 30 min. Simultaneously 0.5 ml. of a 0.05 % solution of fructose was similarly treated. After rapid cooling the solutions were titrated with $N/200$ KMnO_4 until the blue colour just disappeared. The amount of fructose present was calculated using standard fructose as a basis. A blank value obtained from fasting blood was subtracted from each result (the assumption being made that normal blood contains no fructose) since the colour so obtained was always greater than that given by the reagent alone.

2. *Diphenylamine method.* 0.2 ml. blood was diluted with water to 0.6 ml. and 0.2 ml. each of 10 % ZnSO_4 and 0.5 N NaOH added. After heating to 80° for 10 min. the mixture was filtered. 1.0 ml. of the filtrate was acidified with 2 drops of 0.1 % acetic acid and was evaporated to 0.4 ml. 0.4 ml. 6 N HCl and 1 drop 20 % alcoholic diphenylamine were added and the tube heated on the boiling water-bath for 15 min. After cooling for 2 min. 1.0 ml. butyl alcohol and 0.2 g. solid $(\text{NH}_4)_2\text{SO}_4$ were added. The tube was stoppered and briskly shaken. The upper alcoholic layer was pipetted off for comparison in the micro-colorimeter. The standard was prepared from suitable dilutions, in fasting blood filtrate, of a stock standard fructose solution, treated in exactly the same way as the blood filtrates.

All the rabbits used were tested to ensure that they gave normal sugar curves when glucose alone was administered and each animal fasted for 24 hours before an experiment.

Total reducing sugar in the blood was estimated by the method of Hagedorn and Jensen. Preliminary experiments showed that in this method fructose had the same reducing power as glucose. Glucose was estimated by difference.

Blood samples were collected by pricking an ear vein and allowing the blood to drip into a watch glass containing oxalate. The specimens were collected as quickly as possible and samples were immediately pipetted into the precipitating reagents. In the injection experiments the sugar solution was warmed to 37° and injected into the marginal ear vein opposite to that from which blood samples were taken. In the feeding experiments the sugar was at first fed by stomach tube but later it was found to be less disturbing to the animal to feed the sugar as a syrupy paste between two pieces of cabbage leaf.

The curves shown below are specimens which approximate closely to the average of the series and are not necessarily the "best" of the series.

RESULTS AND DISCUSSION.

1. *Fructose injection.*

Injected fructose was found to disappear from the blood stream at about the same rate as glucose (Fig. 1).

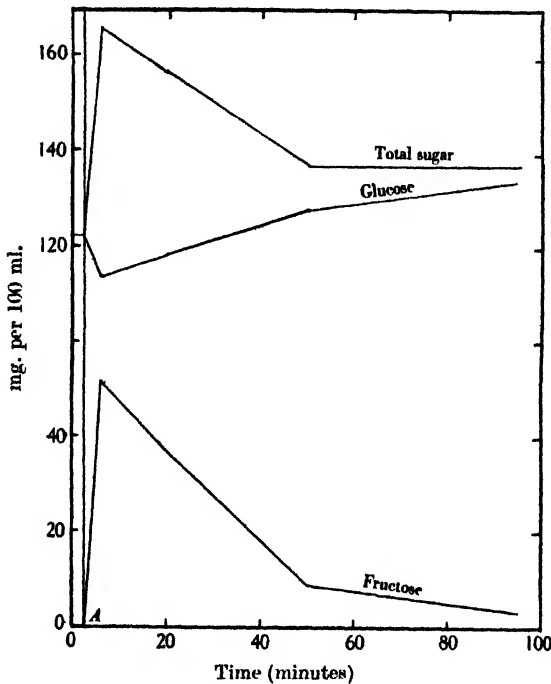


Fig. 1. 0.25 g. fructose injected at A.

When fructose alone was injected there was invariably found to be a slight fall (about 10 mg./100 ml.) in the blood glucose. The tables given by Corley [1929] show a similar slight fall in some cases but this is in contradiction to the findings of several other authors, notably Van Creveld and Ladenius [1928] and Harding *et al.* [1933] who found that the rise in total reducing sugar after fructose ingestion could not wholly be accounted for by the fructose present in the blood.

It is possible, and in view of other evidence distinctly probable, that fructose causes the secretion of insulin by the pancreas, and that this insulin is responsible for the fall in glucose (see section 3).

2. *Fructose ingestion.*

The effect of ingestion of 1.25 g. fructose was irregular. In some cases there was no rise in the total reducing sugar of the blood whilst in others there was a delayed rise. In a few cases a curve somewhat similar to that obtained by feeding glucose but with a much smaller rise in total sugar was obtained. This is in agreement with the work of Kimball [1932], Harding *et al.* [1933] and Corley [1929]. Such a rise in fructose was accompanied, as before, by a slight fall in glucose.

3. Ingestion of glucose followed by injection of fructose.

The hypothesis that fructose stimulates the secretion of insulin was tested by feeding glucose and injecting fructose 15 min. later while the glucose curve was still rising. The injection of fructose was found to convert the rise in glucose into an immediate fall and the glucose curve returned to the fasting level much more quickly than when glucose was ingested alone (Fig. 2).

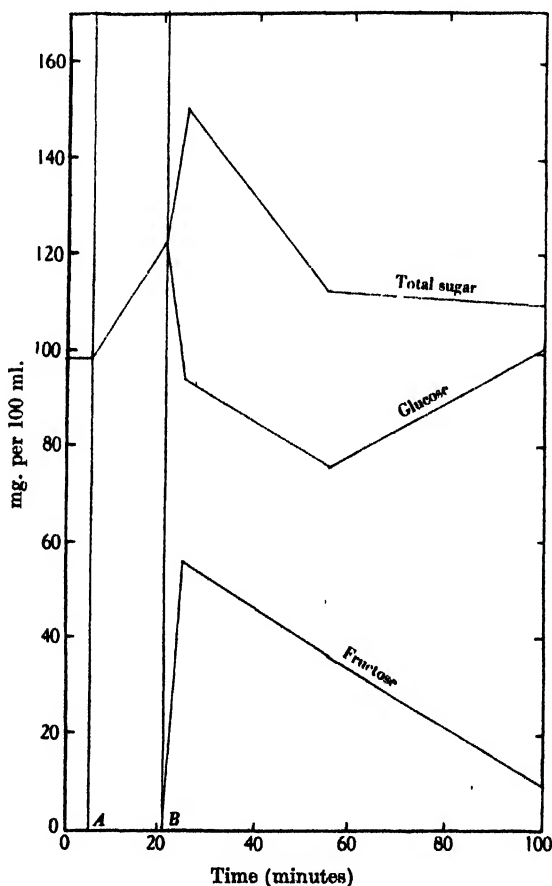


Fig. 2. 1.25 g. glucose fed at A. 0.25 g. fructose injected at B.

The fructose disappeared at the same rate as when it alone was injected. Obviously the injection of fructose has caused an immediate acceleration of the disappearance of glucose from the blood and it is most probable that it has done so by stimulating the pancreas to produce insulin.

4. Ingestion of glucose followed by injection of insulin.

The glucose curve obtained in the previous experiment could be closely reproduced by feeding glucose and injecting insulin (intravenously) instead of fructose

(Fig. 3). The rise in the glucose curve was converted into an immediate fall and it appears exceedingly probable that the fall in the glucose curve in the previous experiment was due to insulin produced under the influence of fructose.

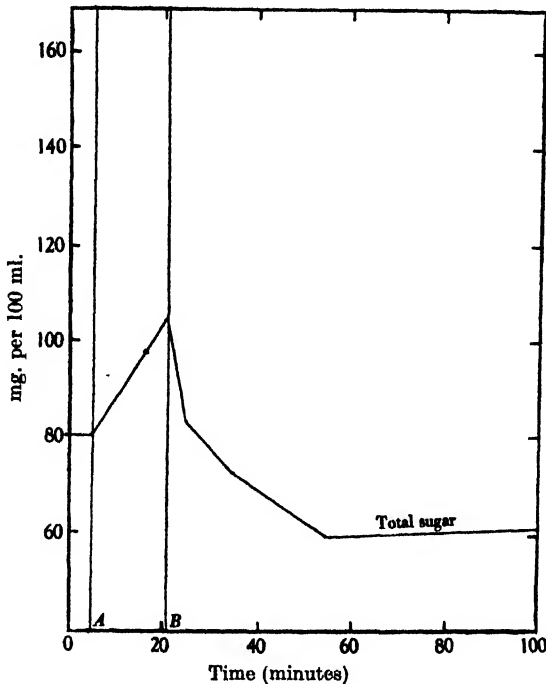


Fig. 3. 1.25 g. glucose fed at A. 0.8 unit insulin injected at B.

5. *Injection of fructose followed by injection of glucose.*

As a variation to previous experiments glucose was injected 4 min. after fructose had been injected. The extra glucose disappeared more rapidly from the blood and the glucose curve returned to the fasting level more rapidly than when glucose alone was injected (Fig. 4). The fructose curve was identical with those previously obtained. We can explain these results by assuming that the injection of fructose stimulated the secretion of a certain amount of insulin which acted on the glucose subsequently injected, causing it to disappear more rapidly than it would otherwise have done. The insulin however had no effect on the rate of removal of fructose from the blood.

This explanation is supported by the fact that the glucose curve shown in Fig. 4 was closely reproduced when insulin was injected along with glucose.

6. *Ingestion of glucose followed by injection of glucose.*

When glucose was injected after glucose had been fed the rise in the total reducing sugar was greater than when fructose was injected after the ingestion of glucose. The extra glucose disappeared rapidly from the blood stream (more rapidly than when glucose was injected or ingested alone) but the fall was less rapid and the return to the fasting level more delayed than when fructose injection followed the feeding of glucose (Fig. 5).

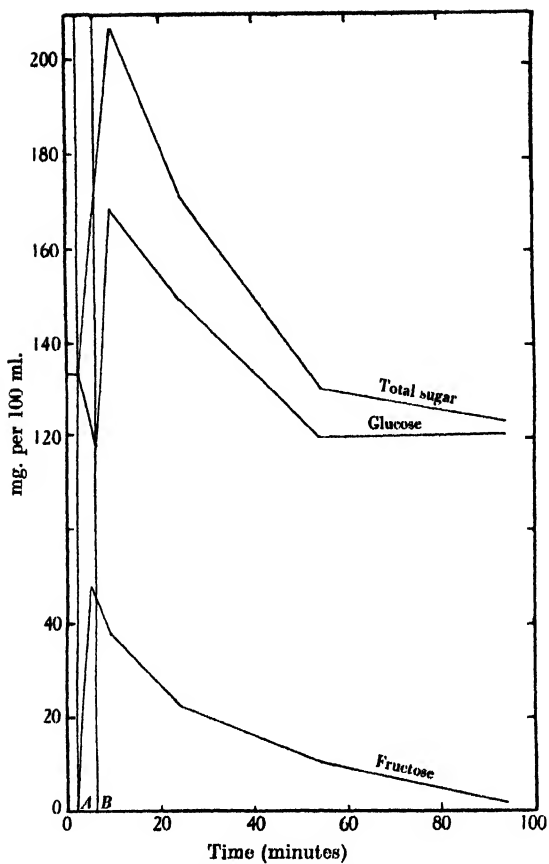


Fig. 4. 0.25 g. fructose injected at *A*. 0.25 g. glucose injected at *B*.

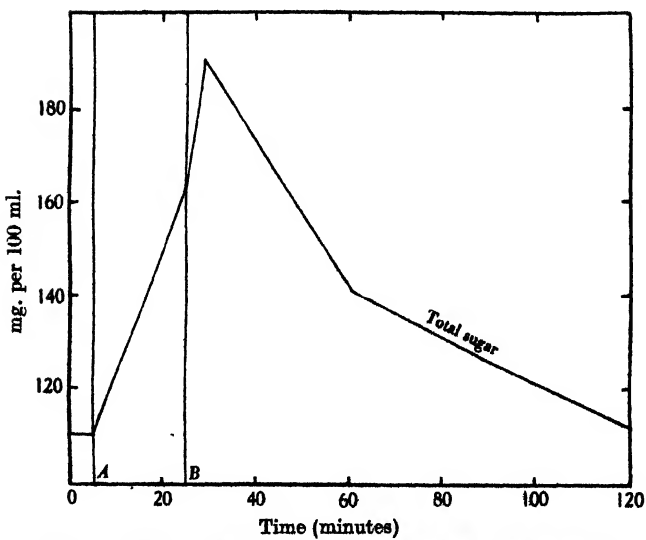


Fig. 5. 1.25 g. glucose fed at *A*. 0.25 g. glucose injected at *B*.

A possible explanation of the higher rise in the total reducing sugar obtained in this experiment as compared with the rise obtained when fructose injection followed glucose ingestion is that fructose is more effective than glucose in stimulating the pancreas to secrete insulin. In the absence of further evidence however it is impossible to arrive at any definite conclusion regarding the relative stimulating powers of the two sugars, since the one sugar stimulates insulin production but does not utilise the insulin so produced while the other both produces and utilises insulin.

7. *Injection of fructose and insulin.*

The intravenous injection of insulin (in doses up to 0.6 unit) simultaneously with fructose was found to produce no effect on the rate of disappearance of fructose from the blood (Fig. 6). This is in agreement with the results of Corley

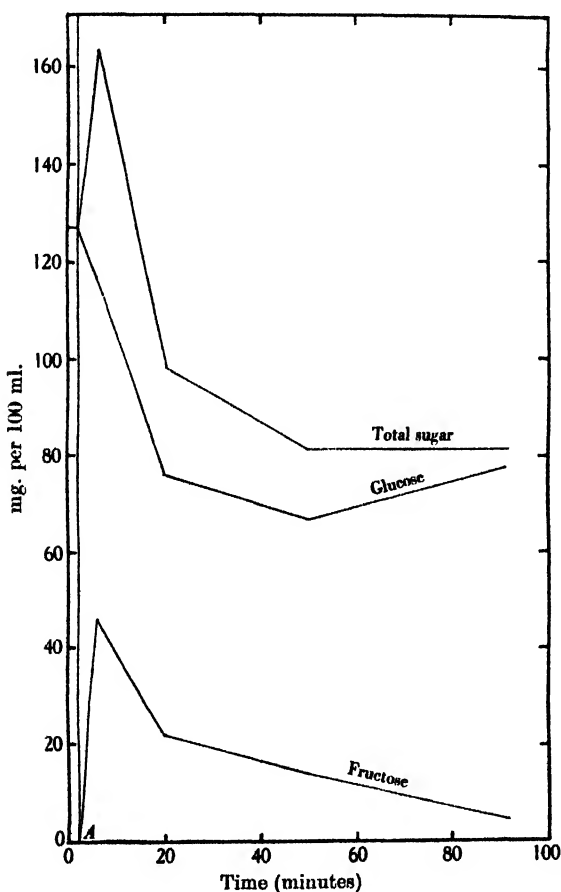


Fig. 6. 0.25 g. fructose and 0.3 unit insulin injected at A.

[1929] who found that fructose could protect rabbits against insulin although the latter had no effect on the rate of removal of the fructose from the blood. Wierzuchowski [1926] also states that insulin has no effect on the rate of removal of fructose.

Cori and Cori [1927, 1, 2; 1928] found that insulin affected the disposal of fructose and that surplus led to an increased oxidation although it did not influence the tolerance for intravenously injected fructose.

All these results are consistent with the hypothesis that the first stage in fructose metabolism is the conversion of fructose into glucose (or some similar substance) and that the rate of this conversion is independent of insulin, whilst the second stage is the disposal of the glucose (or similar substance) and is controlled by insulin.

The question of the metabolism of fructose is of course by no means settled by these experiments. They do indicate very strongly however that in all probability fructose is converted into glucose or some similar substance (*e.g.* hexosephosphoric acid) independently of insulin. It is likely that this conversion takes place in the liver in view of the known importance of the liver in fructose metabolism. The results also suggest that fructose stimulates the secretion of insulin although the insulin so produced can be required only in the second stage of fructose metabolism. The results appear to us to be of interest in connection with the known tolerance of diabetics for fructose since, if the pancreas is still capable of producing insulin at all, administration of fructose will (*a*) provide a sugar which is converted into glucose at a rate small enough for the deficient supply of insulin to deal with and (*b*) stimulate the flagging production of insulin.

SUMMARY.

1. Two methods for the estimation of fructose in small amounts of blood are described.
2. These methods have been used to investigate the fate of ingested and injected fructose in rabbits.
3. The results obtained suggest that fructose stimulates the pancreas to secrete insulin.
4. Insulin however has been found to have no effect on the rate of removal of fructose from the blood and it is therefore suggested that the metabolism of fructose takes place in two stages, (*a*) the conversion, independently of insulin, of fructose into an unknown substance which is most probably glucose, (*b*) the disposal of this substance under the influence of insulin.

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LXX. THE MECHANISM OF DEGRADATION OF STARCH BY AMYLASES.

I. NATURE OF THE EARLY FISSION PRODUCTS.

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OHLSSON [1926; 1930] concluded from experiments on the action of the "dextrinogenamylase" and "saccharogenamylase" of malt on starch that the former enzyme breaks starch into progressively smaller molecules until maltose is produced, whereas the latter enzyme detaches maltose molecules successively from the starch. This conclusion was based mainly on the relative permanence of the starch-iodine reaction during digestion by the respective enzymes at equal saccharifying concentrations. The argument is not wholly reliable, since the nature of the starch-iodine reaction is not understood, and it is not certain that colour is a function of molecular dimensions. Therefore an investigation has been made of the actual reducing products present in starch partly degraded by these and similar enzymes. As the dextrinogenamylase of malt is an α -amylase [Ohlsson, 1930] in the sense that the products of its action on starch exhibit mutarotation in the α -sense [Kuhn, 1925], this enzyme will be referred to herein as α -malt-amylase. Similarly the saccharogenamylase, a β -amylase, will be termed β -malt-amylase.

The investigation is concerned with hydrolytic products at a comparatively early stage in the degradation of starch. A preliminary experiment showed that the velocity of hydrolysis of soluble starch by α -malt-amylase in low concentration became inconstant at 15–20 % hydrolysis whilst at 25 % hydrolysis the iodine reaction had changed to red. Therefore stages corresponding to 15, 20 and 25 % hydrolysis were selected as likely to be informative. β -Amylase from malt and from ungerminated barley showed no colour transition until the hydrolysis was too far advanced to be of use.

EXPERIMENTAL.

Lintner soluble starch was used. Its reducing power expressed as percentage maltose was 3.9 (iodimetric titration) and 1.53 (Bertrand); $[\alpha]_D^{20} + 198.4^\circ$.

The malt-amylases were prepared from a highly diastatic Canadian malt by the method of Ohlsson [1930]. For the α -enzyme, an aqueous extract was heated to 70° for 15 min. with stirring, filtered cold, precipitated in 60 % alcohol, freed from alcohol, dissolved in water and filtered clear after adjustment of the reaction to the desired value of p_H . For the β -enzyme, the aqueous extract was brought to p_H 3.3 at 0° for 15 min., restored to p_H 4.6, warmed to normal temperature and further treated as above. Both enzymes could be stored at -5° and p_H 6.4 without appreciable loss of activity. The amylase of ungerminated barley was prepared by precipitation of 20 % alcoholic extract in alcohol *etc.* Commercial pancreatin was used as a source of pancreatic amylase.

Control tests under the conditions of reaction employed in the experiments, indicated the absence of any maltase activity from all of the amylase preparations.

To 480 ml. lots of 5.6 % (dry weight) starch were added respectively:

- | | |
|---|-----------------|
| (1) α -malt-amylase equivalent to 10 g. malt | } at p_H 4.6. |
| (2) β -malt-amylase equivalent to 8 g. malt | |
| (3) β -(barley)amylase equivalent to 10 g. barley | |
| (4) α -(pancreatic)amylase equivalent to 0.1 g. pancreatin at p_H 6.8. | |
- Total volume 500 ml.

Each reaction was allowed to proceed at 25° until iodimetric titration of an aliquot indicated the percentage hydrolysis desired. The remaining reaction mixture (usually about 450 ml.) was then poured slowly into 300 ml. of boiling water, boiling and stirring being maintained. The mixture was concentrated to 500 ml. and 20 ml. withdrawn to determine afresh the total reducing power. 250 ml. of each were now evaporated to 50 ml. and dried on a water-bath with washed, ignited kieselguhr until syrupy. The mass was suspended in 500 ml. of boiling absolute alcohol, decanted and the residue extracted under reflux 5 times with 150 ml. of 95 % alcohol. The final extracts contained no reducing matter. The combined extracts were evaporated to dryness, treated with water and re-evaporated thrice to remove alcohol, aldehyde *etc.* and finally dissolved in water and brought to 25 ml. The residue, after freeing from alcohol *etc.*, was heated with water in an autoclave at 120° for 30 min. The filtrate was finally contained in 500 ml. of aqueous solution.

Analyses of these fractions gave the results shown in Table I.

Table I. *Analysis of amylolytic hydrolysates.*

	α -Amylase				β -Amylase		
	Malt			Pancreas	Barley		Malt
Apparent maltose in hydrolysate:							
Percentage theory	25.6	20.6	19.3	14.8	24.3	14.8	20.6
In portion fractionated, g.	3.41	2.31	2.33	2.03	3.27	1.98	2.44
Alcohol-soluble (sugar) fraction:							
Maltose (iodimetric), g.	1.36	0.59	0.60	0.87	3.11	1.40	2.01
Maltose (polarimetric), g.	2.71	—	—	1.18	3.12	1.41	—
Maltose osazone	Small amounts				Large amounts		
Alcohol-insoluble (dextrin) fraction:							
Dry weight, g.	9.39	9.58	8.44	11.60	9.28	10.03	—
Reducing power (Bertrand):							
As maltose, %	23.0	20.3	21.1	11.4	4.5	7.1	—
$[\alpha]_D$	187°			191°	189°	195°	
Iodine reaction	Violet	Red-violet		Blue-violet	Blue	Blue	
Retrogradation after 7 days	None	None	None	None	Retrogradation		
Osazone	No crystalline osazone formed						

Velocity of fermentation of alcohol-soluble fractions.

Aliquots of the alcohol-soluble fraction possessing reducing powers equivalent to 0.5 g. of maltose were fermented at 30° with 8 g. of pressed brewer's yeast in 50 ml. total volume [Hopkins and Roberts, 1935; 1936]. The rates of fermentation at intervals and the total CO₂ evolved up to the time of practical cessation are recorded in Table II.

Table II. *Rates of fermentation of alcohol-soluble fractions (mg. CO₂/min.).*

Fraction	Apparent maltose g.	Min.								Total CO ₂ mg.
		5	10	15	20	25	30	40	50	
Pure maltose	0.500	7.2	6.2	6.1	5.9	5.7	5.6	3.3	0.8	232
α (25.6% hydrolysis)	0.546	7.2	4.3	4.0	3.2	2.5	2.5	1.2	0.5	157
β (24.3% hydrolysis)	0.477	7.0	6.4	6.0	5.9	5.5	4.9	2.0	0.2	205
Pure maltose	0.500	7.4	6.8	5.9	5.2	4.6	4.3	3.0	1.2	228
α (14.8% hydrolysis)	0.545	7.0	5.3	4.8	4.1	3.5	3.0	2.1	1.2	181
β (14.8% hydrolysis)	0.516	7.0	6.7	5.9	5.2	5.0	4.4	3.0	1.6	224

DISCUSSION.

The alcoholic extracts from the β -amylase hydrolysates contained maltose in quantity nearly equivalent to the total reducing value of the original hydrolysates. It was identified by its specific rotation, reducing power, osazone and in certain instances by the characteristic fermentation by brewer's yeast under conditions described by Hopkins and Roberts [1935; 1936]. The β -amylase products fermented throughout at approximately the same rate as pure maltose of approximately the same concentration, the total CO₂ evolution confirming the identity. After alcohol extraction the "dextrin" residue, dispersed in water at 120°, exhibited properties (Table I) characteristic of slightly degraded starch.

Hydrolysis by α -malt-amylase yielded an alcoholic extract which contained maltose (confirmed by osazone). However, the reducing power was only half of that which would correspond with the specific rotation, assuming the usual relationship between R and $[\alpha]_D$ for maltose. No further fractionation or analysis of this obvious mixture was attempted. It fermented at rates much lower than maltose, although employed in slightly higher concentration. From the known (unpublished) relationship between velocity of fermentation and initial maltose concentration (compare Hopkins and Roberts [1935] for glucose), it is possible to deduce that the fermentation products of α -amylase (apparent maltose 0.55 g.) actually contained not more than 0.25 g. maltose. The total CO₂ from the α -products is fictitiously high owing to slow fermentation of dextrins. This persisted long after pure maltose or the corresponding β -products had been completely fermented; nevertheless the total CO₂ clearly indicates that only a fraction of the extractable reducing power was due to maltose. In one experiment pancreatic α -amylase was used with similar results.

If α -amylases break down starch or the major part of it into reducing dextrins, then, assuming the reducing groups of the latter to exercise reducing power equal to corresponding groups in maltose, the 20–25% stage of hydrolysis (as percentage maltose) means that the products have an average chain-length of about 8–10 glucose units. Those, including maltose, of shorter length are present presumably in the alcohol-soluble fraction and apparently all slowly fermentable. The more complex ones, of longer chain-length, which appear in the other fractions give a violet colour with iodine and possess appreciable reducing powers whereas the corresponding fraction from the products of β -amylase gives a blue iodine reaction, exhibits relatively little reducing power and in general character rather resembles starch than dextrin.

SUMMARY.

1. α -Malt-amylase and pancreatic amylase produce some maltose in the early stages of hydrolysis of soluble starch. The major part of the reducing material formed has a higher $[\alpha]_D$ than maltose and the alcohol-soluble fraction

is rather slowly fermented by brewer's yeast. The conclusion of Ohlsson that these enzymes split starch initially into dextrins is confirmed.

2. β -Malt-amylase and the amylase of ungerminated barley produce maltose in the early stages of starch hydrolysis in an amount virtually equivalent to the reducing power. The remaining material resembles amylose or starch. The view that β -amylases remove successive molecules of maltose from the starch molecule is confirmed.

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LXXI. THE MECHANISM OF DEGRADATION OF STARCH BY AMYLASES.

II. KINETICS OF ACTION OF α - AND β -AMYLASES ON STARCH, ITS COMPONENTS AND PARTIAL DEGRADATION PRODUCTS.

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VAN KLINKENBERG [1934] suggested that α -amylases promote hydrolysis of " α -starch" (which is a fraction resembling Wijsman's "erythrogranulose" or Baker's " α -amylodextrin" and constitutes about 36 % of the starch). β -Amylases were correspondingly specific for β -starch (the remainder). Hydrolysis beyond 36 % in presence of α -amylase was explained by the supposed slow transformation of β - into α -starch. Now starch in solution contains certain distinct components which may be fractionated by electrodialysis yielding respectively amyloamylose (blue with iodine) and erythroamylose (red with iodine) [Samec and Mayer, 1921]. Amyloamylose is hydrolysed by pancreatic (α -), barley (β -) and unfractionated malt (mixture) amylases relatively easily and completely, but erythroamylose only with difficulty [Samec and Waldschmidt-Leitz, 1931, this paper Table I]. Thus Van Klinkenberg's so-called α - and β -starches are not identical with amylo- and erythro-amyloses, though they might represent fractions of the erythroamylose, which constitutes at least 60 % (recovered by us) to 83 % (Samec) of the total starch.

α -Malt-amylase does not selectively hydrolyse the erythrogranulose or α -amylodextrin fraction of starch [Hanes, 1935: this paper Table I]. In this communication are reported observations on the kinetics of hydrolysis promoted by α - and β -amylases or by their mixtures. The substrates included starch, amyloamylose, erythroamylose (components of starch), α -amylodextrin and certain fission products of the enzyme actions. α -Amylodextrin may be regarded either as a component of the original starch or as a fission product of the action of β -amylase.

EXPERIMENTAL.

Substrates.

Lintner soluble starch as described in Part I. α -Amylodextrin was prepared from the soluble starch by the action of alcohol-precipitated barley amylase at p_H 4.6 and 50° by a method closely resembling that described by Baker [1902]; yield 33 %, $[\alpha]_D^{20} + 188^\circ$, reducing power (R) 1 % as maltose. *Glycogen* (Pfanzstiehl), $[\alpha]_D^{20} + 185^\circ$, R 0.7 %. *Achroodextrin* was prepared from soluble starch by the action of commercial (B.D.H.) pancreatin at p_H 6.8 and 25° until the reducing power corresponded to 51 % as maltose, the dextrin being precipitated in and washed by 80 % boiling alcohol until the washings were free from reducing power and finally dried over H_2SO_4 at 60°; yield 46 %, $[\alpha]_D^{20} + 160.6^\circ$, R 32.8 %.

Amyloamylose and erythroamylose were prepared by repeated electro dialysis at 220 v. of potato starch (autoclaved at 120°) [Samec and Mayer, 1921], using parchment membranes and platinum electrodes.

Table I. *Hydrolysis of soluble starch, α -amylodextrin and glycogen by α -malt-amylose, β -barley-amylose and mixtures of the two.*

Amylase	Equivalent weight of barley or malt per 100 ml. g.	Substrate	Dry weight of reaction mixture g.	Hydrolysis as percentage of theoretical maltose							
				Minutes				Hours			
				7	20	40	90	210	8	48	
α	1.25	Soluble starch	2.00	3.06	8.74	16.3	27.4	35.5	41.8	54.0	
	2.5			6.20	15.9	25.8	32.8	40.6	47.0	64.7	
	5.0			11.7	25.4	31.8	37.4	46.3	52.7	76.6	
β	1.25	Soluble starch	2.00	2.16	4.91	9.0	18.3	34.3	47.7	55.6	
	2.5			3.54	8.94	17.5	33.0	50.1	56.4	58.3	
	5.0			6.09	16.7	31.9	48.6	56.7	60.0	64.0	
	13.0			10.5	36.8	49.0	—	—	—	—	
$\alpha + \beta$	1.25 α	Soluble starch	2.00	5.08	13.5	23.7	43.4	62.6	—	76.3	
	1.25 β			—	—	—	—	—	—	—	
	1.25 α			9.90	26.3	47.8	66.3	70.6	—	79.0	
	5.00 β			—	—	—	—	—	—	—	
	1.25 α			18.5	49.4	66.6	—	—	—	—	
	13.0 β			—	—	—	—	—	—	—	
α	5.0 α	α -Amylo-dextrin	2.00	9.25	23.3	39.3	60.6	69.8	—	80.8	
	5.0 β			—	—	—	—	—	—	—	
	5.0 α			—	—	—	—	—	—	—	
α	1.25	α -Amylo-dextrin	2.00	3.27	8.45	14.7	20.3	25.6	29.8	42.1	
	2.5			6.27	14.0	19.1	23.9	28.6	33.7	50.1	
	5.0			10.9	18.8	22.9	26.6	31.7	37.5	60.6	
β	1.25	α -Amylo-dextrin	2.00	—	—	1.1	—	—	1.6	2.3	
	5.0			—	1.0	2.0	—	—	2.0	2.5	
$\alpha + \beta$	1.25 α	α -Amylo-dextrin	2.00	4.2	11.2	18.5	29.2	35.4	—	49.4	
	1.25 β			—	—	—	—	—	—	—	
	1.25 α			6.15	16.8	26.2	32.5	36.7	—	50.8	
	5.0 β			—	—	—	—	—	—	—	
α	2.5 α	Glycogen	2.00	8.03	18.9	27.9	34.1	38.5	—	62.0	
	2.5 β			—	—	—	—	—	—	—	
	2.5 α			—	—	—	—	—	—	—	
α	1.25	Glycogen	2.00	1.53	3.80	6.12	10.3	14.4	20.0	33.9	
	2.5			2.98	6.11	9.53	13.7	19.1	25.3	45.6	
	5.0			5.00	9.15	12.9	18.1	24.2	31.0	68.3	
β	1.25	Glycogen	2.00	—	—	—	4.79	8.16	12.1	20.5	
	2.5			1.13	—	—	8.16	12.6	18.1	25.7	
	5.0			1.97	—	—	12.9	18.9	23.8	29.1	
$\alpha + \beta$	1.25 α	Glycogen	2.00	3.12	7.72	11.9	22.7	33.2	—	54.0	
	1.25 β			—	—	—	—	—	—	—	
	1.25 α			—	—	—	—	—	—	—	
	5.0 β			5.17	13.2	20.7	30.4	38.8	—	56.7	
Hydrolysis by β -amylase (barley) of amyloamylose and erythroamylose.											
β	11.8	Amylo-amylose	0.256	19.8	40.5	64.0	89.0	—	94.8	96.8	
β	22.2	Erythro-amylose	1.60	38.0	46.2	48.4	49.9	—	53.8	57.7	
β	22.2	Soluble starch	1.60	45.4	54.3	56.6	57.8	—	60.5	63.4	

The enzymes were prepared as described in Part I, and were free from maltase. The quantities used are expressed in terms of the equivalent quantities

Table II. *Hydrolysis by β -amylase (barley) of α -amylodextrin previously degraded to 20% of theoretical by α -malt-amylase.*

Amylase	Equivalent weight of barley per 100 ml. g.	Substrate	Dry weight per 100 ml. of reaction mixture g.	Results as percentage of theoretical maltose					
				Minutes				Hours	
				7	30	90	210	8	23
β	0.6	Degraded dextrin	2.20	2.70	8.62	11.4	12.9	13.8	14.0
	5.0		2.20	10.6	12.6	14.3	14.8	15.4	16.0
β	0.6	(Original dextrin	2.20	0.2	0.8	1.0	1.5	-	2.3
	5.0		2.20	0.2	1.1	1.7	3.2	-	-

Table III. *Hydrolyses of achroodextrin by α - and β -malt-amylase.*

Amylase	Equivalent weight of malt per 100 ml. g.	Substrate	Dry weight per 100 ml. of reaction mixture g.	Results as percentage of theoretical maltose				
				Minutes		Hours		
				20	90	4	8	24
α	2.5	Achroo-dextrin	0.69	6.87	9.74	12.7	16.3	22.4
	5.0		0.69	7.91	11.5	14.8	17.3	23.9
	2.5	Starch	2.0	17.2	32.9	41.2	—	56.3
β	2.5	Achroo-dextrin	0.69	13.1	18.0	21.9	24.9	32.2
	5.0		0.69	15.5	20.5	24.7	28.5	38.6
	2.5	Starch	2.0	13.9	51.2	—	—	64.9

of malt or barley from which they were prepared. Exps. 1-5 (Table I) were performed with the same enzyme preparations, which were stored in a refrigerator and showed little change in activity.

All reactions were performed at p_H 4.6 (0.015 *N* acetate) and 25°, the enzyme solutions having been brought to p_H 4.6 by the addition of acetic acid. The progress of hydrolysis was followed iodimetrically, controls for the reducing power of the enzyme preparation and of all substrates being deducted. There is doubt whether, in the case of "non-reducing" substrates, such as starch, α -amylodextrin, glycogen and the amyloses, this deduction is justified, but it seemed desirable in this instance to adopt a uniform procedure throughout. All results are expressed as percentage of maltose theoretically equivalent to the quantity of substrate used. In some experiments control hydrolyses of starch were performed. In that reported in Table IV the appropriate quantities of maltose were added to the controls. The results in Table IV were calculated on the total concentration of carbohydrate in the reaction mixture less that of maltose equivalent to the reducing power. To indicate percentages in terms of the original starch the final percentage hydrolysis stated should therefore be

Table IV. *Hydrolysis by β -malt-amylase of starch previously degraded to 20% hydrolysis by (a) α -amylase, (b) β -amylase and the enzymes destroyed.*

Amylase	Starch previously degraded by	%	Minutes					Hours	
			7	20	40	90	210	8	24
β (dil.)	α -amylase	2.0	2.44	5.95	10.8	22.9	40.0	44.8	48.6
	β -amylase	2.0	2.05	5.19	9.93	18.6	27.3	31.0	36.9
	Control	2.0	2.21	5.83	10.9	21.3	36.3	43.4	48.3
β (conc.)	α -amylase	2.0	38.4	44.3	46.0	49.9	53.5	58.7	—
	β -amylase	2.0	28.7	34.3	37.1	43.5	49.5	55.5	—
	Control	2.0	34.2	41.8	44.5	50.6	54.5	60.1	—

multiplied by $(100-x)/100$, where x is percentage of the original conversion. The values of x for the four hydrolyses quoted in Table IV were respectively α 21.1, β 21.1, control 21.1, α 21.9, β 18.7, control 21.6.

DISCUSSION.

The kinetics of hydrolysis of starch by α - and β -amylases separately, confirm the results reported by Van Klinkenberg and by Hanes. When employed together in low concentrations, the α - and β -amylases function additively. However, with higher concentration, particularly of the β -enzyme, the rate of hydrolysis in the early stages exceeds the sum of the component rates (Table I). The effect on starch may be partly attributed to the presence of α -amylopectin (but see also discussion of Table IV). The observation that the limit of hydrolysis with mixed amylases is only 10–12% greater than with single amylases of similar total activity and never approaches 100%, suggests that at least half of the starch constitutes a substrate common to both enzymes.

Greater activity was shown in the late stages and higher limiting hydrolyses attained than in the experiments of Hanes at 35°, or of Van Klinkenberg at 40°. Inactivation of the enzyme is the probable explanation. The β -amylase by itself exercised a negligible effect on α -amylopectin, but acting in conjunction with the α -enzyme it evidently hydrolysed some of the early fission products formed by the latter, since the rates of hydrolysis by the combined enzymes greatly exceeded the sums of the component hydrolyses [see also Hanes, 1935]. This conclusion was confirmed by allowing β -amylase to attack α -amylopectin previously hydrolysed to 20% by α -amylase, after destruction of the latter enzyme, with controls (Table II). An appreciable further hydrolysis took place.

Results similar to those with α -amylopectin were obtained with glycogen, which in this respect behaves as if intermediate in character between starch and α -amylopectin (Table I).

Achroodextrin, the maltose-free product of hydrolysis of starch to 50% by pancreaticin, was more rapidly hydrolysed by β - than by α -amylase (of about equal activities on starch). The limit, about 40% in the case of β -amylase, leaves 60% of the dextrin unhydrolysed; this is equivalent to 30% of the original starch, and probably corresponds to a large part of the α -amylopectin fraction of the latter. β -Amylase hydrolyses α -amylopectin, previously slightly degraded by the α -enzyme, to about 15% (Table II), leaving perhaps 70% of the dextrin, equivalent to 28% of the original starch. This agrees in quantity with the undegraded residue of the achroodextrin. The achroodextrin probably contains a high proportion of α -amylopectin, the hydrolysis of which by the pancreatic (α -) amylase has not progressed far. That it should succumb more rapidly to the action of β - than α -amylase is in agreement with the greatly reduced rate of starch hydrolysis promoted by the latter enzyme at the 50% stage, as compared with that of the β -enzyme when of equal initial activity (see Table I).

The products of the actions on starch of the α - and β -amylases to the 20% stage can be understood from the results of certain experiments described in Part I. When β -amylase acted on these products and a control of starch + maltose and enzyme concentration was the limiting factor, the three substrates were hydrolysed at approximately the same rates, except in so far as modified by the approach of the limit. With very high concentrations of the β -amylase, the starch previously degraded by the α -enzyme was more rapidly attacked than the others. This may be due either to the higher molecular concentration of substrate,

which had in this case been broken up into dextrans by the α -enzyme, or to the relative absence of maltose, an inhibiting agent. On the whole there is no clear evidence that, at this stage of the hydrolysis, the dextrinous fission products of the α -amylase are more susceptible to the action of β -amylase than is starch.

The conclusions to be drawn from these experiments and discussion are as follow.

(1) Both α - and β -amylases readily attack and hydrolyse completely the amyloamylose component (about 20 %) of starch, and a portion of the erythroamylose component.

(2) Both enzymes hydrolyse much the same fraction, 60 %, of the starch; but whereas the remainder (α -amylodextrin) is almost if not completely resistant to the β -amylase, it can be, with difficulty, hydrolysed by the α -enzyme.

(3) α -Amylodextrin, the hydrolysis of which has been commenced by the α -amylase, can be further degraded to a certain extent by the β -amylase. The α -enzyme holds the key to further progress.

(4) In degrading starch to the 50 % stage, the α -enzyme does so mainly at the expense of the portion which is other than amylodextrin, since action on the residue by β -amylase still leaves about 30 % of the original starch unattacked.

SUMMARY.

The velocities of hydrolysis of soluble starch, amyloamylose, erythroamylose, α -amylodextrin, achroodextrin and glycogen by α -malt-amylase and β -malt- or barley-amylase have been measured. The results confirm those of previous workers. A mixture of the α - and β -amylases in low concentration functions additively in hydrolysing starch. A mixture with β -amylase in high concentration promotes hydrolysis of starch at a rate greater than the sum of the component rates. Mixtures at either concentration hydrolyse α -amylodextrin and glycogen at rates greater than the sums of the component rates.

Achroodextrin, a fission product of the action of α -amylase, is hydrolysed more rapidly by β -amylase than by α -amylase. α -Amylodextrin previously degraded by α -amylase to about 20 % hydrolysis, is (after destroying the α -enzyme), hydrolysed by β -amylase. The residues left when α -amylodextrin is attacked successively by α - and β -amylases and when achroodextrin is hydrolysed by β -amylase correspond in each case to about 28–30 % of the original starch. Amyloamylose is readily hydrolysed by β -amylase to nearly 100 % maltose, whereas erythroamylose is degraded with greater difficulty than starch, and to a limit within 60 %.

The suggestion of Van Klinkenberg of selective hydrolysis of different components of the starch by the respective amylases is not supported by the evidence. It appears that both amylases hydrolyse the same (non- α -amylodextrin) portion of the starch, amounting to about 60 %. Only the α -amylase can attack the α -amylodextrin portion, which however is not appreciably degraded by the enzyme until the hydrolysis of the starch is fairly advanced.

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LXXII. THE MECHANISM OF DEGRADATION OF STARCH BY AMYLASES.

III. MUTAROTATION OF FISSION PRODUCTS.

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KUHN [1925] concluded from measurements of optical rotation that the amylases of pancreas and of *Aspergillus oryzae* apparently yielded α -maltose as a primary fission product of starch, whereas malt amylase produced β -maltose. Subsequently Ohlsson [1930] found by the same method that the two amylases of malt fractionated by his method [1926, 1930] also behaved as α - and β -amylases respectively, and that of ungerminated barley as a β -amylase.

The attempt to accommodate these findings with the conception of starch as being composed of a single type of chemical molecule has led to certain questions. (1) Are these primary fission products actually α - and β -maltose, or are they other bodies? (2) Are the observations of Kuhn to be explained on the basis of changing rotation of the starch itself, i.e. that the substrate as distinct from its fission products is mutarotating owing to one of two or more components being selectively hydrolysed? (3) Does dispersed starch consist of two chemically different molecules one containing α -glucosidic linkages only, the other including β - as well as α -glucosidic linkages? In this case the phenomenon observed by Kuhn would be an essential attribute of the substrate and not of the enzyme, it being assumed that α -amylase preferentially attacked the former and β -amylase the latter type of molecule. Van Klinkenberg's [1934] hypothesis includes some such basis as this. There are in any case however powerful *a priori* reasons for rejecting any hypothesis that starch contains two types of molecule so different chemically as those containing only α -linkages and those containing both α - and β -linkages.

Of the above questions, (1) and (2) have been dealt with to some extent. The reducing materials formed in the early stages of hydrolysis are, from the α -enzyme some maltose, but mainly dextrans, from the β -enzyme, maltose only [Freeman and Hopkins, 1936, 1]. α - and β -amylases attack most readily the same 60% fraction of starch. There is no evidence of substantial selective hydrolysis [Freeman and Hopkins, 1936, 2]. To help to answer question 3 it was necessary to devise experiments to ascertain whether the actions of the amylases give rise to their characteristic mutarotations respectively, irrespective of the physical fraction of starch used as substrate. Kuhn used as substrates amylose and an electrodialysed fraction of starch, whilst Ohlsson used only the latter. Neither of these fractions represents starch as a whole. In the experiments reported here we have allowed forms of α - and β -amylases to act on Lintner soluble starch, amyloamylose, erythroamylose (Samec), glycogen α -amylo-dextrin (Baker) and α -amylodextrin previously partly degraded by α -amylase. The use of α -amylodextrin was considered particularly important since this is the portion of starch which resists degradation by β -amylase. The mutarotation was observed sometimes directly, as by Kuhn, but usually on a product obtained

by filtration through a specially prepared filter designed to pass only smaller molecules. Dialysis took too long for successful observations. Filtration was adopted, firstly, to remove the enzyme, unchanged substrate and less degraded products, with the idea of observing if the mutarotating substances were among the more degraded reaction products; secondly, because, soon after the commencement of the reaction with α -malt-amylase, a hazy substance forms, which soon prevents polarimetric observation but later flocculates. (This was also observed by Hanes [1935].) On adding to alkali to complete the mutarotation, a flocculum is formed, the removal of which by filtration reduces the optical rotation. Some of the "mutarotation" in the α -sense observed in Exp. 3, and by previous workers, must be due to this removal from solution of optically active material. Filtration before polarimetric observations obviates this.

EXPERIMENTAL.

The substrates used and the enzymes prepared were as described by Freeman and Hopkins [1936, 2]. The particular specimen of soluble starch used had been selected on account of its transparency in solution, and for the sake of uniformity, was used throughout the work recorded previously. All percentages of substrates refer to dry weight. No buffer salts were added when velocities of mutarotation were to be measured; elsewhere a concentration of 0.015 *N* acetate was maintained. The values of $k \times 10^3$ were calculated, in Exp. 1 A, from the 1st and 7th, 2nd and 8th readings *etc.* and in the other experiments in a corresponding manner. The alkali used to complete mutarotation was Na_2CO_3 .

The technique of filtration through a ferric phosphate gel will be described in a later communication.

Exp. 1. Enzyme, pancreatic (α) amylase; substrate, soluble starch. 200 ml. 2.68% starch + A, 15 ml., B, 30 ml. of 5% pancreatin. Temp. 20°, p_{H} 6.8, filtrations at A, 28–36 min., B, 17–26 min.; 0.1 ml. of 5% HgCl_2 added to 50 ml. of filtrate to arrest enzyme action.

Table I. *Optical rotation of filtrates (2 dm.) and velocity constant of mutarotation.*

Time from commence- ment of readings min.	A			B		
	Rotation (angular degrees)	Mutarotation	$k \times 10^3$	Rotation (angular degrees)	Mutarotation	$k \times 10^3$
0	9.44	- 0.82	6.32	7.16	- 0.52	6.25
5	9.38	- 0.76	6.23	7.13	- 0.49	6.87
10	9.33	- 0.71	5.96	7.08	- 0.44	6.03
15	9.27	- 0.65	5.70	7.05	- 0.41	5.65
20	9.23	- 0.61	5.88	7.03	- 0.39	—
25	9.18	- 0.56	(5.48)	—	—	—
30	9.15	- 0.53	—	6.97	- 0.33	—
40	—	—	—	6.93	- 0.29	—
50	9.08	- 0.46	Mean 6.02	6.90	- 0.26	Mean 6.20
60	8.98	- 0.36	—	—	—	—
70	8.93	- 0.31	—	—	—	—
80	8.90	- 0.28	—	—	—	—
∞	8.62	—	—	6.64	—	—
0	8.65 (alkali added)	—	—	6.62 (alkali added)	—	—
<i>R</i> of filtrate, hydrolysis %	63.4			45		
<i>p</i> _H of filtrate	6.72			6.84		
Original reaction mixture, hydrolysis %:						
(1) At time of filtration	64			58		
(2) Final (24 hours)	70			71.3		

Exp. 2. Enzyme barley (β) amylase; substrate soluble starch. A, 200 ml. of 3.5% starch + 40 ml. of amylase (\equiv 80 g. barley). B, 200 ml. of 2.68% starch + 30 ml. amylase (\equiv 60 g. barley). Temp. 20°, p_H 4.6. Filtered at 10–16 min., then 0.1 ml. of 0.85% $AgNO_3$ added to arrest enzyme action. Read from 20 min. onwards.

Table II. *Optical rotation of filtrates (2 dm.) and velocity constant of mutarotation.*

Time from commence- ment of readings min.	A			B		
	Rotation (angular degrees)	Mutarotation (angular degrees)	$k \times 10^3$	Rotation (angular degrees)	Mutarotation (angular degrees)	$k \times 10^3$
0	8.28	+0.52	6.25	5.84	+0.37	6.05
5	8.31	+0.49	5.84	5.87	+0.34	3.35
10	8.34	+0.46	4.90	5.90	+0.31	5.64
20	8.41	+0.39	—	5.93	+0.28	—
30	8.45	+0.35	Mean 5.66	5.96	+0.25	Mean 5.68
40	—	—	—	6.00	+0.21	—
45	8.49	+0.31	—	—	—	—
∞	8.80	—	—	6.21	—	—
0	8.80 (alkali added)	—	—	6.25 (alkali added)	—	—
50	8.80 (alkali added)	—	—	6.25 (alkali added)	—	—
<i>R</i> of filtrate, hydrolysis %	41.6	—	—	37.4	—	—
n_H of filtrate	4.88	—	—	4.52	—	—
Original reaction mixture, hydrolysis %:						
(1) At time of filtration	47	—	—	47.4	—	—
(2) Final (24 hours)	55.2	—	—	59.1	—	—

Exp. 3. 75 ml. of 2.68% starch + 50 ml. of α - or β -malt amylase (\equiv 100 g. malt). Temp. 20°, p_H 4.6.

Table III. *Optical rotations and mutarotations of reaction mixtures and filtrates.*

Time		Rotation (1 dm.)	Rotation after alkali addition (angular degrees)	Muta- rotation	Iodine reaction	Conversion (iodimetric) % maltose
min.	sec.					
α -Amylase						
0	0	(2.94)*	(2.94)*	—	Blue	0
1	0	3.04	—	—	—	—
2	0	3.09	—	—	—	—
2	10	3.06	2.75	-0.31	Red	25.8
7	40	—	2.66	—	Yellow	32.1
18	45	—	2.63	—	Colourless	37.9
42	30	—	2.58	—	„	42.7
1440	0	—	2.18	—	„	83.6
22	0	filtrate 2.21	1.96	-0.25	„	22.9
26	0					
β -Amylase						
0	0	(2.97)*	(2.97)*	—	Blue	0
3	0	2.71	2.77	+0.06	—	26.7
7	30	2.40	2.60	+0.20	Blue	40.3
17	30	2.35	2.46	+0.11	Blue-violet	54.7
40	30	2.35	2.38	+0.03	„	57.5

* Calculated.

Exp. 4. A, 100 ml. of 3.00% α -amyloextrin + 20 ml. α -amylase (\equiv 40 g. malt). B, 4.00% α -amyloextrin, hydrolysed by α -malt-amylase to 20.7%

hydrolysis, the enzyme destroyed and 100 ml. of the product equivalent to 2.84 % of undegraded α -amylodextrin + 20 ml. barley amylase (\equiv 40 g. barley). C, 100 ml. of 3.00 % α -amylodextrin + 20 ml. α -malt-amylase (\equiv 40 g. malt) + 20 ml. barley amylase (\equiv 40 g. barley). Temp. 20°, p_H 4.6. Results in Table IV (4 A, 4 B, 4 C). Percentage hydrolysis in the reaction mixtures is indicated in col. 6, the figures in brackets referring to the filtrates, and all in terms of the substrate concentration in the original reaction mixtures.

Exp. 5. 100 ml. of 3.0 % glycogen + 20 ml. A, α -malt-amylase; B, barley amylase. Temp. 23°, p_H 4.6. Results in Table IV (5 A, 5 B).

Exp. 6. 60 ml. of 1.95 % amyloamylose + 10 ml. A, α -malt-, B, barley amylase. Temp. 20°, p_H 4.6. Results in Table IV (6 A, 6 B).

Exp. 7. 60 ml. of 1.95 % erythroamylose + 10 ml. A, α -malt-, B, barley amylase. Temp. 20°, p_H 4.6. Results in Table IV (7 A, 7 B).

Table IV. *Progress of reactions and mutarotations of filtrates.*

	Time of filtration min.	Time of reading min.	Rotation (1 dm.)	Rotation after alkali addi- tion (1 dm.) (angular degrees; corrected)	Muta- rotation (1 dm.)	Conversion (iodimetric) maltose %
<i>α-Amylodextrin</i>						
4 A	—	5	—	—	—	16.2
		12	—	—	—	20.2
	8-15	17	2.92	2.78	-0.14	(11.5)
		35	—	—	—	25.3
	33-38	40	2.92	2.79	-0.13	(15.8)
	—	8 (hr.)	—	—	—	50.1
4 B	—	7	—	—	—	10.6
	10-12	14	3.85	3.95	+0.10	(6.6)
	—	30	—	—	—	12.6
	30-32	34	3.74	3.81	+0.07	(7.5)
	—	90	—	—	—	14.3
4 C	—	5	—	—	—	27.0
		12	—	—	—	33.0
	8-13	15	2.77	2.71	-0.06	(22.2)
		35	—	—	—	37.8
	33-38	40	2.61	2.55	-0.06	(24.7)
	—	4½ (hr.)	—	—	—	49.9
<i>Glycogen</i>						
5 A	10-20	22	3.93	3.80	-0.13	(20.5)
	32-38	40	4.51	4.43	-0.08	(26)
5 B	10-14	16	4.11	4.21	+0.10	(20)
	30-34	36	3.88	3.97	+0.09	(25)
<i>Amyloamylose</i>						
6 A	10-12	14	1.01	0.96	-0.05	—
6 B	10-12	14	1.11	1.17	+0.06	—
<i>Erythroamylose</i>						
7 A	10-12	14	2.19	2.04	-0.15	—
	30-32	34	2.02	1.89	-0.13	—
7 B	10-12	14	1.94	2.01	+0.07	—
	30-32	34	1.73	1.86	+0.13	—

DISCUSSION.

The filtered solutions contained very little enzyme, in Exp. 1 A none. Mercuric chloride (Exp. 1 B) and silver nitrate (Exps. 2 A and 2 B) were added to prevent further enzyme action. Traces of chlorides in the pancreatic amylase,

prevented the use of silver in the case of Exp. 1 B. Control tests showed that these salts in the concentrations used effectively checked enzyme action but had no perceptible effect on the rate of mutarotation of maltose.

α -Amylase and β -amylase, with all the substrates used, gave filtrates which exhibited α - and β -mutarotations respectively, the unimolecular velocity constants of which approximated to those of β -maltose under the same conditions. The initial fission products of α -amylase are reducing and mutarotating "dextrins" and some α -maltose, the later products, *e.g.* at 60–70 % hydrolysis as in Exp. 1, mainly α -maltose to which the observed velocity constants refer. The velocity constants of mutarotation of pure β -maltose to equilibrium have been determined by us over a wide range of temperature and p_H in the absence of salts, the relevant results being summarised in Table V.

Table V. *Unimolecular velocity constants of mutarotation at 20° (unbuffered).*

	$k \times 10^3$			
p_H	4.52	4.88	6.72	6.84
β -maltose to equilibrium	5.00	5.00	—	5.48
α -amylase fission products	—	—	6.02	6.20
β -amylase fission products	5.68	5.66	—	—

In the presence of acetate buffers, 0.04*N*, the values of the constant for β -maltose were substantially higher, *e.g.* 5.8 instead of 5.0 at p_H 4.66, and 11.16 instead of 5.4 at p_H 6.81. The ions, Na^+ and Cl^- , which were present in small concentrations in the reaction mixtures have no catalytic effect on mutarotation, but unavoidable traces of other ions may account for the values of $k \times 10^3$ being high.

SUMMARY.

Experiments recorded, taken in conjunction with those in Parts I and II, indicate that the α - and β -types of amylase, as typified by those of the pancreas, malt and ungerminated barley, respectively attack starch in different ways. Whilst each enzyme degrades most easily the same 50–60 % of the starch, including the whole of the amyloamylose portion, the α -enzyme alone can attack the α -amylodextrin portion. Comparatively little of the fission products arises from degradation of the latter during the first 50–60 % of the hydrolysis of starch by the α -enzyme.

Further differences in the mode of action are evident. In the initial stages of hydrolysis, α -amylase produces reducing dextrins and a little maltose, giving α -mutarotation, whilst β -amylase produces β -maltose as almost the sole reducing product. Later the reducing products resulting from the action of the α -enzyme include increasing proportions of α -maltose, as was indicated by the observations of Kuhn and confirmed with certain precautions in this work. The velocities of mutarotation to equilibrium of the filtered reaction products of advanced hydrolyses with both enzymes agree reasonably well with those observed for β -maltose under similar conditions. The possibility that the observations of Kuhn and of Ohlsson were due, in the cases of one or both enzymes, to mutarotation of unattacked substrate, one component of which had been selectively hydrolysed, is excluded.

The sense of mutarotation of the reaction products is a characteristic of the enzyme and is independent of the substrate employed, whether it be soluble

starch, one of its components or glycogen. In short α - and β -amylases, attacking the same material, produce respectively α -dextrins (and later α -maltose) and β -maltose.

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LXXIII. THE ESTIMATION OF SUGARS IN THE LEAF OF THE MANGOLD (*BETA VULGARIS*).

- I. METHODS FOR THE ESTIMATION OF FRUCTOSE, GLUCOSE AND SUCROSE.
- II. THE USE OF BASIC LEAD ACETATE, CHARCOAL AND YEAST TO AVOID INTERFERENCE BY SUBSTANCES OTHER THAN SUGARS.
- III. NOTE ON THE APPLICATION OF COPPER REAGENTS TO UNCLARIFIED EXTRACTS.
- IV. TESTS FOR THE PRESENCE OF RAFFINOSE, MALTOSE, GALACTOSE AND PENTOSE.

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I. METHODS FOR THE ESTIMATION OF FRUCTOSE, GLUCOSE AND SUCROSE.

METHODS based on the reducing powers of sugars are generally considered to be the most suitable for the analysis of leaf extracts and have almost completely superseded those based on optical activity or other physical properties. Two types of oxidising agent are commonly used for sugar estimation, namely, reagents of the alkaline copper tartrate group and those of the ferricyanide group. In the present study of the sugars of the mangold leaf both these types have been used.

Micro-reagents were employed since they are sufficiently accurate, rapid in use and are the only reagents which are suitable for methods of sugar estimation which involve the treatment of leaf extracts with yeast.

Many of the micro-reagents of the copper group are unsuitable for the analysis of plant extracts because they contain potassium iodide as stabiliser. Iodides hold cuprous oxide in solution and permit back-oxidation by the air [Shaffer and Somogyi, 1933]. Since the degree of back-oxidation is constant for given concentrations of sugar and iodide this is not a great disadvantage in the analysis of pure sugar solutions, except for very low concentrations of sugar which may give no apparent reduction at all. In the analysis of plant extracts, however, a serious disadvantage arises. It is impossible, for example, to estimate the reducing power of enzyme blanks or the residual reducing power of extracts after treatment with yeast, since, in both cases, the reducing power is sufficiently small to fall within the zone of iodide-induced insensitivity.

A modification of the Harding and Downs copper reagent.

Harding and Downs [1933] have developed an iodide-free reagent which they applied by heating 2 ml. of sugar solution for 10 min. with 2 ml. of reagent. With the minor variation that 5 ml. each of sugar solution and reagent were used, their method was employed in most of the earlier investigations reported here, using the factors for glucose and fructose recorded in Table I.

The Harding and Downs reagent contains sodium bicarbonate and sodium carbonate in the ratio of 5 to 4. With reagents of this type the heating period of 10 min. used by them is sufficient to bring about maximum oxidation of fructose, but the oxidation of glucose increases with time up to almost 15 min. [Shaffer and Somogyi, 1933]. As a result, when using a 10-min. period, small variations in the time or conditions of heating cause varying degrees of reduction of the reagent by glucose; and it was found impossible to reproduce the factors published by Harding and Downs or even to reproduce exactly the same factor from day to day.

The procedure was therefore modified by increasing the time of heating to 15 min. In addition oxalates were omitted from the solution at the time of heating since they serve no useful purpose at this stage and tend to lower the reduction of the reagent [Shaffer and Somogyi, 1933], and slight alterations were made to allow of a more convenient preparation of the reagent.

The following solutions were used in the modified reagent:

- | | | |
|----|--|--------------|
| A. | 23 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per litre. | |
| B. | 35 g. anhydrous sodium bicarbonate | } per litre. |
| | 28 g. anhydrous sodium carbonate | |
| | 17 g. Rochelle salt | |
| | 1 g. potassium iodate | |
| C. | 10 g. potassium iodide | } per litre. |
| | 19 g. potassium oxalate | |
| D. | <i>N</i> sulphuric acid. | |
| E. | Standard thiosulphate solution (0.01 to 0.02 <i>N</i>). | |

Care is needed in the preparation of Solution B. The bicarbonate is dissolved separately at a temperature below 25° , and the solution is then added to a solution of the carbonate, tartrate and iodate which is also prepared in the cold. The whole is then diluted to a litre and kept in a cool place; at 1° the solution keeps indefinitely. The copper reagent is prepared by mixing 2 volumes of Solution A with 5 volumes of Solution B. The mixed solutions keep for 1 day only, and fresh reagent should be mixed daily.

For analysis 5 ml. of sugar solution are mixed with 5 ml. of reagent ($2A + 5B$) in a 6×1.25 inch (150×32 mm.) pyrex hard glass test-tube. This is plugged with non-absorbent cotton-wool or covered with a glass lid. The tube is then heated in a vigorously boiling water-bath for the specified time, after which it is placed in cold water for about 2 min. to cool the contents to about 25° . 5 ml. of Solution C are now added and well mixed and are followed immediately by 5 ml. of Solution D. The whole is well shaken and allowed to stand several minutes to ensure complete reaction, after which the liberated iodine is estimated by titration with thiosulphate, using starch as indicator. Blank determinations are made by substituting water for the sugar solution.

Estimations are conveniently carried out in batches of six. The tubes are heated and cooled together, but after adding Solution C to a tube it must be

flowed by Solution D before proceeding to the next tube. The titration with thiosulphate is carried out after the iodine has been liberated in all the tubes. The amount of reagent reduced by glucose and fructose in 15 min. is recorded in Table 1. The reducing power of invert sugar may be taken as intermediate between those of glucose and fructose, and for other mixtures of the two sugars proportional to the amounts of each present.

The two columns in Table 1 for fructose in the presence of 0.221 % KI are used for the determination of fructose after the oxidation of glucose by hypochlorite under conditions to be described later. A heating period of 10 min. has been chosen here, since it is sufficient for fructose alone.

Table 1. *The reduction when 5 ml. of the Harding and Downs copper reagent or of the modified reagent are heated with 5 ml. of sugar solution*

Concentration of sugar g. per 100 ml.	Reduction in ml. 0.01 N thiosulphate			Reduction in ml. 0.01 N thiosulphate		
	Harding and Downs's reagent			Modified Harding and Downs's reagent		
	Glucose	Fructose	Fructose with 0.221 % KI	Glucose	Fructose	Fructose with 0.221 % KI
	Heating period (min.)	Heating period (min.)	Heating period (min.)	Heating period (min.)	Heating period (min.)	Heating period (min.)
	10	10	10	15	15	10
50		6.52	6.27	6.88	6.66	6.41
20	4.12	4.26	4.02	4.53	4.33	4.08
10	2.05	2.05	1.83	2.20	2.12	1.89
5	1.02	1.00	0.79	1.06	1.03	0.81
2.5	0.51	0.49	0.29	0.52	0.51	0.32
1						0.13
1.25	0.25			0.26	0.25	

The ferricyanide reagent.

The modification of Hane's method as described by Widdowson [1931] was used. The factors given for the various sugars were found to be reproducible and were therefore adopted.

The effect of added reagents on the sugar factors.

Various substances which possess no reducing properties of their own have the power of changing the apparent oxidation of sugar by either the copper reagent or the ferricyanide reagent. Tests were therefore made on all the substances which are usually added during the preparation of leaf extracts for analysis.

In the presence of 4 % sodium chloride it was found that glucose was underestimated by the copper reagent and slightly overestimated by the ferricyanide reagent. The relative errors were greater at low concentrations of sugar.

The following figures were obtained for the copper reagent: taken 15.9, 1.59 mg.; found 14.7, 1.03 mg., and for the ferricyanide reagent: taken 15.9 mg.; found 16.6 mg.

From these results it is evident that hydrochloric acid should not be used to invert sucrose or destroy fructose except under special circumstances, and its use is only permissible in small amounts with high concentrations of sugar.

Neither reagent is affected by 2 % potassium sulphate or 2 % sodium acetate. When acetates are present, however, it is desirable to use a little extra acid prior to the thiosulphate titration. Disodium hydrogen phosphate in large amounts affects the copper reagent, and with reagents such as that of Harding and

Downs, which are poor in tartrate, may even cause a precipitate to be formed during heating. It should be used only at a concentration below 0.1 %. Acids increase and alkalis decrease the reducing power towards copper. With the modified copper reagent an excess of one drop of *N* sulphuric acid in the 5 ml. sample used for analysis will increase the reduction by about 0.5 to 1.0 %.

The estimation of fructose in the presence of glucose.

The literature dealing with the various methods which have been used to estimate fructose in the presence of other sugars has recently been summarised by Schroeder and Herrmann [1934] and there is no need to refer to it here. The accuracy of most of the available methods even when applied to pure sugar solutions seems open to doubt and only that using hypiodite appears to give trustworthy results.

Hypiodite (alkaline iodine) oxidises glucose (or other aldoses) to the corresponding acid, but scarcely affects fructose, and it may therefore be used to estimate glucose directly. Used in this way however it is quite unsuitable for analysis of leaf extracts since the reagent is reduced to a very large extent by many leaf constituents. The reaction may also be used indirectly for the estimation of fructose by oxidising glucose with hypiodite, removing excess of iodine and then determining fructose by means of an ordinary copper reagent. This procedure was first suggested by Kolthoff [1922] as a qualitative test for fructose and was subsequently adopted by Kruisheer [1929] for quantitative estimation of fructose in urine, using a copper reagent. Phillips Mason [1933] also adopted the indirect procedure, using the Shafer-Hart micro-copper reagent for estimating the fructose; their method, however, is inaccurate as no allowance was made for the effect of iodides.

This indirect method of fructose estimation was adopted here, using the following procedure. Into a 25 ml. graduated flask are measured 4–5 ml. of a sugar solution. This should contain 3–5 mg. of glucose (or, in the case of leaf extracts, its equivalent of glucose and hypiodite-reducing substances other than sugar). To this are added 2 ml. of an iodine solution which contains 8.47 g. of iodine and 16.7 g. of potassium iodide per litre. The contents of the flask are then rotated and 1 ml. of 0.167 *N* NaOH is added very slowly, drop by drop. When the neck of the flask is washed down, if necessary, by about 1 ml. of water and the flask stoppered. The reaction is now allowed to proceed for 2 hours at 15–20°C, effective temperature control being obtained by precooling all solutions. These conditions for the hypiodite-oxidation approximate, except as regards volume, to those used by Archbold and Widdowson [1931] for the direct estimation of glucose.

After 2 hours the solution is acidified with 1 ml. of 0.25 *N* sulphuric acid and the excess iodine quantitatively removed by sodium sulphite, using a single small drop of 1 % starch solution as indicator. The sodium sulphite solution should be freshly prepared and about 0.02–0.04 *N*; it is conveniently added from a burette to which is attached a capillary which is sufficiently long to reach down the neck of the flask. The solution is then neutralised with 1 ml. of 0.23 *N* NaOH, and the volume made up to 25 ml. 5 ml. of this are used to estimate fructose.

If the copper reagent is used after the treatment with hypiodite, the factors given in Table I for fructose in the presence of 0.221 % potassium iodide should be employed. This is the concentration of iodide which is present if the conditions given above are fulfilled.

Since the reagents added during the course of the estimation have a reducing power towards the copper reagent it is unnecessary to make a blank

estimation for the whole process. This was demonstrated by adding a small known quantity of sugar to bring the reduction outside the zone of iodide-induced insensitivity. The blank reduction value is therefore obtained by heating the copper reagent with a solution of approximately 0.221 % potassium iodide. This is necessary since iodides prevent the small autoreduction of the reagent, although the difference between a water blank and an iodide blank is usually small. Greater differences than 0.02 ml. of 0.01 *N* thiosulphate were never recorded.

Owing to the manner in which iodides lower the apparent reducing power of sugar towards the copper reagent, the method cannot be used when the ratio of fructose to glucose is less than about 0.05. The amount of sugar which can be treated with hypiodite depends entirely on the amount of glucose present, and with very low ratios of fructose to glucose the amount of fructose may be undetectable. The gluconic acid formed by the oxidation of glucose with hypiodite has no reducing power towards the copper reagent.

With the ferricyanide reagent the normal fructose factors may be used, iodides having no effect. There is invariably a small reduction (equivalent usually to 0.06-0.10 ml. of 0.01 *N* thiosulphate) due to the various reagents added during the estimation. It is therefore essential that a blank estimation, by treating water instead of sugar solution with iodine and alkali, should be made for the whole process.

No allowance was made for the small destruction of fructose by hypiodite. Bailey and Hopkins [1933] have found that when fructose is oxidised by hypiodite, oxalic acid and, presumably, *d*-erythronic acid are produced. Complete destruction of 1 g. of fructose would reduce 5.64 g. of iodine on this basis. In investigating the reduction of iodine by fructose under the conditions which have been adopted here Archbold and Widdowson [1931] found a maximum reduction of 0.017 g. of iodine per g. of fructose. Under these conditions therefore the maximum amount of fructose destroyed is 0.017 g. per 5.64 g. or only 0.3 %.

Gluconic acid reduces ferricyanide slightly and allowance must be made for it. The reduction is equivalent to 0.05 ml. of 0.01 *N* thiosulphate for every mg. of (oxidised) glucose in the 5 ml. sample used for estimation.

Since the above conditions of oxidation (a 2-hour period at 1°) require an adequate control of temperature which it may not always be possible to obtain, a trial was made with an oxidation period of 10 min. at room temperature (18°), which conditions approximate to those used by Hinton and Macara [1924] for the direct estimation of glucose. Using the copper reagent the results with a mangold-leaf extract were the same as those obtained by oxidation at 1°. It is probable that any other conditions which have proved suitable for the direct estimation of glucose could be used for the indirect estimation of fructose. Any error from the slight tendency for hypiodite to oxidise fructose would probably be considerably smaller in the case of the indirect method. For whereas 1 g. of fructose, on complete destruction, reduces the equivalent of 5.64 g. of iodine, 1 g. of glucose is known to reduce only 1.41 g.; in a mixture of the two sugars, therefore, any reduction of the hypiodite by fructose would cause an error in the indirect method which is only a quarter as great as the corresponding error in the direct estimation of glucose.

The estimation of sucrose.

In view of the effect of chlorides on the sugar reagents, invertase was used in place of hydrochloric acid to hydrolyse sucrose. Invertase is very reactive towards the ferricyanide reagent, 1 mg. of a British Drug Houses preparation

being found to have a reducing power equivalent to 0.190 mg. of invert sugar (the corresponding figure towards the copper reagent is only 0.018 mg.). In addition the reducing power towards ferrieyanide of a mixture of sugar and invertase is somewhat less than the sum of the reducing powers of the sugar and of the invertase taken separately. Consequently sucrose may be underestimated, since accurate allowance for the presence of invertase cannot be made by the use of a blank determination using water instead of sugar solution.

By inverting rather concentrated sugar solutions with small amounts of enzyme, this error can be reduced to negligible proportions. The concentrated solutions are diluted prior to analysis. A solution containing about 0.2 % of total sugar was allowed to react at 38° with invertase at a concentration of 0.01 %. Lead-clarified leaf extracts were buffered with acetate to p_H 4.7-4.8; other solutions were unbuffered. A reaction period of about 4 days was used. This long period was used to give a large margin of safety; with many active commercial preparations of invertase as little as an hour may be sufficient. Finally the solution was diluted to a sugar concentration of less than 20 mg. per 100 ml. and analysed. Under these conditions the reducing power of the invertase is reasonably small towards the ferrieyanide reagent and negligible towards the copper reagent..

II. THE USE OF BASIC LEAD ACETATE, CHARCOAL AND YEAST TO AVOID INTERFERENCE BY SUBSTANCES OTHER THAN SUGARS.

It has long been recognised that the use with plant extracts of reagents estimating sugars by their reducing power is complicated by the presence of substances, other than sugar, also possessing reducing power. To overcome this difficulty "interfering" substances have been removed by precipitants such as basic lead acetate or by adsorbents such as charcoal. Alternatively, sugar has been removed by treatment with yeast, the difference in reducing power before and after the treatment being taken as a measure of reducing sugar.

When these methods have been applied to leaf extracts widely different estimates of sugar have usually been obtained, and it has been impossible to decide which, if any, of the many methods is trustworthy. A method of ~~assuring~~ accuracy for the determination of the true sugar content is much to be desired. The choice of such a method should be based solely on the criterion of ~~true~~ worthiness, whether it be suitable for routine analysis being a matter of ~~quite~~ secondary importance. Procedures which, on account of their rapidity, simplicity and applicability to dilute solutions, meet the needs of routine ~~analysis~~ should be considered only after the development of a standard procedure, by comparison with which the accuracy of more expeditious methods may then be assessed.

With the object of devising a procedure suitable for determining the true sugar content of leaf extracts, a detailed study has been made of clarification by basic lead acetate.

This reagent lends itself in greater measure to control and standardisation than any other, and it is therefore most likely to furnish the required standard method. At the same time attention has also been paid to the use of che

and of yeast; the latter in particular has shown promise of providing an accurate, though somewhat empirical method of analysis.

The mangold (*Beta vulgaris*) has been used for this investigation. This plant has previously been studied by Campbell [1912], by Davis *et al.* [1916] and by others.

THE PREPARATION OF LEAF EXTRACTS AND THE METHODS OF ANALYSIS.

Three extracts of mangold leaves were prepared, from two large samples collected in October 1933 from the Rothamsted Experimental Station, and in September 1934 from Slough. Extract I was prepared from mature leaves of the 1933 collection, Extract II from immature leaves of the 1934 collection (approximate size 10×6.3 cm.): water content 84.8%. Extract III was prepared from immature leaves of the 1934 collection which had been kept in the dark for 24 hours with their petioles in water. During this period the water content rose to 86.2%.

For the preparation of the extracts the petioles were removed, 2-4 hours after collection in the case of Extracts I and II, and the laminae killed by boiling alcohol. They were then twice extracted for several hours with six times their weight of boiling 75% alcohol under a reflux condenser, and the alcohol was removed under reduced pressure at a temperature below 33° . In view of the large quantity of material employed for Extract I the water-soluble fraction of this residue could not conveniently be taken up directly. Instead, the mass was treated with water and ether, after which the water fraction was separated, washed several times by shaking with ether, freed from ether and preserved. On account of the method of preparation, results from this extract cannot be calculated in terms of original leaf weight. The residues from Extracts II and III were taken up in water directly and filtered. The filtrates were brought up to a volume such that 1 ml. of the extract was equivalent to 1 g. of the fresh weight of the lamina. All three extracts were nearly free from suspensions of wax and other solid matter. They were preserved with toluene, and toluene-saturated solutions were used in all cases except those involving treatment with yeast.

In these extracts sucrose was estimated as the increase of reducing power after hydrolysis with invertase, fructose as the residual reducing power after oxidation of glucose by hypiodite and glucose as the difference between the total reducing power and fructose. Parallel estimations for the three sugars were carried out with ferricyanide and copper reagents. The modified Harding and Downs reagent was used for Extracts II and III and the unmodified reagent for Extract I (see p. 458).

The detection of interference by substances other than sugar.

No systematic attempt appears to have been made to study quantitatively the degree of removal by the various clarification processes of substances interfering with the sugar estimations. Absence of colour in the cleared solution which has often been taken as an indication of the efficiency of clarification cannot be considered an adequate guide. Apart from the fact that colour is dependent on dilution, the deeply coloured substances are, in general, readily precipitated by lead, leaving an almost colourless solution which may still contain considerable amounts of interfering substances.

The sensitiveness of hypiodite towards substances other than sugar appears to offer a means of detecting the presence of interfering substances, by a comparison of the estimates of glucose obtained by the use of this reagent with those obtained by the use of a copper reagent, after different clarification processes.

Clarification can only be considered efficient when the hypiodite estimate is lowered to a value which is in close agreement with that obtained by copper. There are two advantages in the use of hypiodite for this purpose. Its oxidising action on substances other than sugar is far stronger than that of copper (v. Table VII), and it is very reactive towards phenolic substances [Gardner and Hodgson, 1909] which are present in considerable amounts in leaf extracts. It is, moreover, relatively sensitive towards substances other than sugar because its oxidising action on glucose is weak. Thus whilst a molecule of glucose absorbs 3.7 atoms of oxygen on oxidation with the Harding and Downs copper reagent, only one atom is absorbed from hypiodite. In consequence even if the interfering substances were to absorb equal quantities of oxygen from the two reagents, the error on the estimate of glucose by hypiodite would be 3.7 times as great as that on the estimate by copper. Taken together these two facts show that agreement between the estimates by hypiodite and copper may reasonably be inferred to indicate the absence of interfering substances.

CLARIFICATION BY BASIC LEAD ACETATE.

The reactions involved during the addition of basic lead acetate to plant extracts, and the detailed method to be followed in its practical application have, hitherto, not been satisfactorily determined. It was therefore decided to study in detail the processes of precipitation and to include a quantitative inquiry into the behaviour of both the basic and the acetate fractions of the reagent.

The estimation of total lead. Total lead was determined volumetrically by the molybdate method. When applied to plant extracts it was found necessary first to separate the lead as sulphide to avoid interference with the end-point by some substance, presumably tannic acid or a similar compound. The sulphide was then dissolved in hot concentrated HCl and prepared for analysis in the usual way.

The estimation of basic lead. The basic fraction of basic lead acetate was determined by estimating the amount of KOH liberated by potassium oxalate. For a qualitative test for base an indicator solution is used containing 25% potassium oxalate and a generous allowance of phenolphthalein. After neutralising with a few drops of dilute acetic acid or NaOH, 1 ml. of the indicator solution is added to 1 or 2 ml. of the solution to be tested. A pink or red colour indicates basic lead, and the test is extremely delicate.

For a quantitative determination of base, lead oxalate is precipitated in a known excess of acetic acid to avoid the formation of basic salts. The solution containing lead base is acidified with 10 ml. of 0.1 *N* acetic acid, the volume brought to about 40 ml. with carbonate-free water, and 2 ml. (excess) of neutral 25% potassium oxalate solution are added. Without filtering, the excess acetic acid is titrated with *N* NaOH. A large amount of phenolphthalein should be used as lead oxalate tends to obscure the end-point. From the difference in the titration figures for acetic acid before and after the precipitation of lead the amount of lead base may be calculated. 1 ml. of *N* NaOH is equivalent to 10.36 mg. of lead as base. The following results were obtained with pure lead oxide and lead acetate: taken 81.0 mg. PbO; found 82.4 mg.; this tendency to overestimate basic lead slightly was repeatedly observed: taken 140.7 mg. $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$; found -0.53 mg. of Pb as base. The negative sign indicates the apparent presence of free acid in the acetate, but the amount is sufficiently small to show that normal lead acetate scarcely interferes with the estimation of basic lead.

The reagents used for clarification.

Normal lead acetate. A 10 % solution.

Standard basic lead acetate. This was prepared by the usual procedure of dissolving litharge and lead acetate in the ratio of 13 to 43.

Monobasic lead acetate. This was prepared by adding 89 g. of litharge to a solution of 151 g. of lead acetate in about 700 ml. of freshly boiled water. After dissolving by gentle boiling, the liquid was cooled, diluted to a litre and filtered.

Dibasic lead acetate. This was prepared by boiling 40 g. of lead acetate and 55 g. of lead oxide for 30 min. in a litre of water. The liquid was then cooled and filtered.

The lead contents of these reagents and their specific gravities are shown in Table II.

Table II. *The specific gravities, p_H , and lead contents of basic lead acetates.*

	Approx. formula	p_H of a 0.005 <i>M</i> solution (Britton and Meek)	Sp. gr.	mg. total Pb per ml.	Mg. basic Pb per ml.	% basic Pb
Standard basic lead acetate	—	6.9	1.248	235.6	74.8	31.7
Monobasic lead acetate	$PbO \cdot Pb(C_2H_3O_2)_2$	7.5	1.176	172.0	81.6	49.2
Dibasic lead acetate	$2PbO \cdot Pb(C_2H_3O_2)_2$	8.0	1.073	73.0	50.2	68.8

Britton and Meek [1932] have determined the p_H of 0.005 *M* solutions of basic lead acetates from the titration of 0.005 *M* lead acetate with NaOH. In the presence of sodium acetate the acidity will be somewhat lower than for the lead salt alone, but the error is negligible. Their value for normal lead acetate is 5.9 and those for the basic lead acetates are shown in Table II. Sommer [1927] gives the percentage of base in the standard reagent as 31.9, and also gives useful information as to the specific gravity of the dibasic salt at different concentrations. This salt is the most basic known [Pick and Ahrens, 1909]. The three basic salts turn litmus blue, and with dilute phenolphthalein give a purple (not red) colour; with more concentrated solutions a purple precipitate may be deposited. These reactions are probably due to the formation of lead compounds with the indicators and cannot be regarded as measures of acidity.

The clarification of Extract I.

A measured quantity of the unneutralised extract was suitably diluted with water or alcohol and precipitated with the required amount of reagent. The liquid was then made up to a known volume and centrifuged, after which an aliquot of the cleared solution was withdrawn and de-leaded with hydrogen sulphide. The whole process was carried out rapidly to minimise any reaction with sugars. Aqueous samples were freed from excess hydrogen sulphide by aeration; alcoholic samples were evaporated under reduced pressure until free from alcohol. Finally the solutions were neutralised, made up to volume and analysed.

Results are shown in Table III. Col. I gives the type of lead reagent, and col. II the percentage of alcohol by volume at the time of centrifuging. Col. III gives the volume in ml. to which 1 ml. of Extract I was diluted before centrifuging. The amount of lead added and the amount remaining in solution are given in cols. IV and V respectively, and the difference between values in these columns gives the amount of lead in the precipitate in terms of total lead (acetate + base). The amount of the basic fraction in solution is shown in col. VI.

Table III. *Analysis of Extract I, showing the estimates for the various sugars at different stages of lead-clarification.*

All weights are in mg. per ml. of Extract I.													
Solution	Clearing reagent	°/o alc. II	Vol. of dil. III	Total Pb added IV	Total Pb in sol. V	Basic Pb sol. VI	React. Pb (max.) VII	η VIII (min.)	Apparent fructose		Apparent glucose		
									Copper	Ferri-cyanide	Copper	Ferri-cyanide	Hypo-iodite
Uncleared	65.5	75.4	44.0	50.9	77.5
"	65.7	76.1	43.9	49.9	77.8
"	65.4	75.8	44.0	50.4	77.7
1	Normal lead acetate	0	12.5	118	93	-	.	.	65.4	70.1	42.2	47.6	66.3
2	"	0	12.5	236	208	-	.	.	65.5	68.8	42.0	46.6	61.9
3	Standard basic lead acetate	0	12.5	111	49	0.0	.	.	62.1	65.7	41.6	43.5	57.2
4	"	0	12.5	138	66	+	.	.	61.3	63.5	41.0	43.5	54.6
12	Dibasic lead acetate	0	12.5	146	8	2.7	123	0.12	48.4	49.4	39.9	40.8	44.9
5	Standard basic lead acetate	70	25	111	21	-	.	.	57.2	61.5	41.0	42.3	57.6
6	"	70	25	138	38	-	.	.	55.4	59.2	41.4	42.3	55.6
7	"	70	25	352	153	0.0	134	0.49
8	Monobasic lead acetate	70	25	327	71	6.4	183	0.40	23.5	25.6	35.1	35.8	44.6
9	Dibasic lead acetate	65	25	277	11	-0.2	213	0.25	19.0	20.8	30.9	31.7	37.6

Apparent sucrose

Copper 34.6
Ferri-cyanide 35.0

Copper 34.2
Ferri-cyanide 35.5

Copper 34.4
Ferri-cyanide 35.3

Copper 34.2
Ferri-cyanide 35.2

The data given in cols. VII and VIII will be discussed later. The remaining columns show the estimates of the sugars by the various methods and require no comment. The first three sets of figures, referring to the unneutralised, unclarified extract, indicate the variability to be expected from repeated experiments. As the analyses were made in May, July and October they further show that the extracts remained stable even though unneutralised.

Clarification in aqueous solution. Solutions 1 and 2 were treated with normal lead acetate in relatively large amounts. After treatment the solutions were acid, as shown by the negative sign in col. VI, and the bulk of the lead remained unprecipitated. The ferricyanide estimate for fructose approaches the copper estimate, which is itself unchanged. The estimates for glucose are all lower, but less than half the difference between the copper and hypiodite estimates for glucose is eliminated, which indicates the inefficiency of the clarification.

To simplify the preliminary discussion of the results obtained with basic lead acetate, it will be assumed that the copper estimate for fructose in the unclarified solution is the true one, and that a reduction of this estimate is due to loss of sugar. This assumption will be substantiated later when with charcoal clarification it will be shown that, in the mangold leaf, estimates of fructose by copper are entirely free from interference by other substances.

Since the evidence in the literature is to the effect that sugar is not lost in the absence of free base, sufficient standard basic lead acetate was added to remove all the acid without leaving any free base, giving Solution 3 in the table. Clarification is however still inefficient, and contrary to expectation fructose is lost.

Solution 4 was prepared by adding sufficient standard basic lead acetate to give maximum precipitation (see p. 468). Free base is now present, more fructose is lost and better agreement between the estimates of glucose, by the various methods, is obtained. Finally in Solution 12 the dibasic reagent was added, also in amount sufficient to give maximum precipitation. The results show a marked improvement, even the hypiodite value approaches the other values for glucose, and there can be little doubt that the bulk of the interfering substances has been removed.

The estimate of sucrose. Estimates of sucrose remained unchanged during clarification by lead in aqueous solution, and it is therefore concluded that, within reasonable limits of error, sucrose may be correctly estimated in unclarified mangold extracts. This result must depend on the absence of substances, such as fructosans, which are affected by invertase, and will presumably not be applicable to all plants.

Clarification in the presence of alcohol. Auerbach and Weber [1925] found that lead salts of acids such as malic, succinic, citric and tartaric are less soluble in 50 % alcohol than in water. It was, moreover, observed that after the removal of the precipitate formed on addition of basic lead acetate to an aqueous mangold extract, a further precipitate could be obtained on addition of alcohol. The possible use of alcohol as an aid to clarification was tested in Solutions 5 and 6, which were clarified in 70 % alcohol with the addition of the same amount and type of basic lead acetate as were used for Solutions 3 and 4 respectively. Although alcohol caused the precipitation of more lead, there was no closer agreement between the estimates for glucose by copper and hypiodite, and judging from the rather high losses of fructose, the extra lead appears to have been precipitated in combination with sugar.

Solutions 7, 8 and 9 were prepared by using the three basic lead acetates in such amounts that almost no free acid or free base was left in solution, and an

amount of alcohol sufficient to give almost maximum precipitation. Clarification was still unsatisfactory and in addition the bulk of the fructose and some glucose also were lost. It is evident that alcoholic solutions should be avoided.

The composition of the lead precipitate. An empirical formula for the lead precipitate may be derived from the data of Table III. For example, of the 353 mg. of lead added to Solution 7 only 199 mg. were precipitated and col. VI shows that neither free acid nor free base was present in solution. All the base in the reagent must therefore have reacted with plant acid, and there may have been some further reaction between the acetate fraction of the reagent and the neutral plant salts (of potassium, calcium *etc.*). Since the standard basic lead acetate contained 31.7% of base, 112 mg. of the lead added were basic and reacted completely. The amount of neutral salt present in the extract was estimated, approximately, from the amount of plant base remaining after igniting the extract. The amount of base in 1 ml. of Extract I was found to be equivalent to 22 mg. of lead. The total amount of lead which has reacted lies therefore between 112 mg. and $112 + 22 = 134$ mg. The latter figure, which is a maximum value since it assumes complete reaction of plant salt, is entered in Table III, col. VII as "reactive lead". The balance of the 199 mg. of lead precipitated, namely 65 mg., has not therefore reacted with plant acid or salt, and must have been precipitated as lead acetate. The precipitate is thus a complex acetate of the type $\text{Pb}(\text{OR})_2 \cdot n\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$, where HOR is plant acid. The value of n in this example is 0.49 (65 divided by 134) and is entered in col. VIII. It is obviously a minimum estimate.

The existence of complex acetates was recorded many years ago by Varrentrap [1840] who obtained them from the lead salts of benzoic acid and "Margarinsäure". A list of complex lead acetates derived from inorganic acids has been compiled by Pick and Ahrens [1909], and more recently Gibson and Matthews [1928] obtained basic complex acetates of the type $\text{Pb}(\text{OR})_2 \cdot \text{Pb}(\text{OH})(\text{C}_2\text{H}_3\text{O}_2)$, where R is the aryl group, by treating phenol, *o*-nitrophenol and *o*-bromophenol with basic lead acetate. This result is particularly important in view of the abundance of phenolic substances in the leaf.

The factors influencing the precipitation by basic lead acetate.

The amount of reagent used. Directions for the use of basic lead acetate generally include the recommendation that it should not be added to excess, since this is destructive to sugar. Whilst this is true, it should be remembered that an excess only increases a loss which begins long before precipitation is complete. A more cogent objection seems to have escaped general recognition, namely that an excess of reagent redissolves its own precipitate. Thus Stammer [1882] found lead sulphate to be soluble in basic lead acetate, and Bachmann [1927] has arrived at the same conclusion for lead succinate. If increasing quantities of reagent are added to a mangold extract, the liquid obtained after removing the precipitate becomes increasingly clear, until a stage is reached when it is almost colourless. The process then reverses and the liquid becomes increasingly coloured, until, with a very large excess of reagent, the precipitate completely redissolves. There is thus a stage of optimum clarification, which has been referred to as the point of maximum precipitation. It is shown by all three basic lead acetates but not by normal lead acetate.

By the use of a centrifuge the amount of reagent required to give maximum precipitation can readily be determined. Basic lead acetate is added in small amounts to a sample of the extract. After each addition the whole is centrifuged, and the process repeated until further addition of lead causes mere cloudiness,

without the formation of a definite precipitate. Great help is given by the physical state of the precipitate; as soon as the point of maximum precipitation is passed, the precipitate ceases to flocculate readily, and after centrifuging for 2 min., at 3000 r.p.m., the supernatant liquid is always somewhat cloudy.

The basicity of the reagent and the acetate content of the precipitate. The complex salt $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{ClPb}(\text{C}_2\text{H}_3\text{O}_2) \cdot 3\text{H}_2\text{O}$ is readily soluble in water [Pick and Ahrens, 1909], whereas the corresponding simple PbCl_2 is not. This observation suggested that the lead precipitate of plant extracts might be rendered more insoluble if the acetate content were reduced by the use of a highly basic lead acetate. Accordingly aliquots of a 70% alcoholic solution of stock mangold extract were treated with standard basic, monobasic and dibasic lead acetates respectively, in amounts which left only traces of free acid or free base in solution. In alcoholic solutions the point when neither free acid nor base is present is also approximately the point of maximum precipitation, so that, by the use of alcohol, the reagents were added over the same range of acidity in all three cases, namely from that of the alcoholic mangold extract to that of normal lead acetate, which latter may be taken to be independent of concentration. The effect of difference in alkalinity of the reagents is thus eliminated, leaving only that of varying ratio of base to acid.

The results for these solutions, numbers 7, 8 and 9 in Table III, show that, with increasing basicity of the reagent, the acetate content of the precipitate (col. VIII) is reduced and that this reduction is accompanied by greater precipitation and more effective clarification. The dibasic reagent is also more effective than the standard reagent in aqueous solution (Solutions 12 and 4), but in this case although the effect of reduced acetate is doubtless important, the final acidity must also play a part.

Sommer [1928] treated molasses with reagents of different basicity and found that increasing basicity led to better clarification until an optimum (approximating to the monobasic acetate) was reached. The reagents were not added to give maximum precipitation, but a constant amount of total lead was used, and all samples were neutralised before treatment, which procedure will be shown to alter the course of the reaction completely (see p. 470). Under these circumstances it is probable that the position of the optimum may be accounted for by the fact that the reagents of higher basicity were used in excess and tended to redissolve the precipitate.

The concentration of the extract. The concentrations of total sugar in the mangold Extract I were about 1 and 0.5%, after dilution prior to clarification, in aqueous and alcoholic solutions respectively. Since the lead precipitate is appreciably soluble, even when the acetate content is reduced by the use of a highly basic lead acetate, it is not possible to clarify efficiently at a dilution suitable for the direct application of micro-methods. With standard basic lead acetate the hypiodite value for glucose was reduced to only 72.1 mg. per ml. of extract at this dilution. Conversely, the use of more concentrated extracts gave somewhat better clarification than that recorded in Table III. If, however, very high concentrations are used, a correction for the volume of the precipitate is necessary, and further so much fructose may be lost that the ratio of fructose to glucose falls below the minimum which is required for the successful application of the copper reagent to the estimation of fructose. Owing to the solubility of the lead precipitate, methods involving the washing of the precipitate cannot be used.

The behaviour of basic lead acetate towards unneutralised solutions. On the addition of a few drops of basic lead acetate to an unneutralised plant extract,

substances such as tannic acid, which form lead salts in the presence of free acetic acid, react with the acetate fraction of the reagent to liberate acetic acid, leaving in solution a little lead as normal or acid salt. The solutions, at this stage, are similar to those treated with normal lead acetate, and there is no danger of sugar loss. With further additions of the basic reagent, substances which are able to react in the presence of free acetic acid are removed, and after a phase where only normal lead acetate is present, the solution contains free base. At some point in this phase fructose losses begin. With still larger additions of reagent the point of maximum precipitation is reached; with dibasic lead acetate, the lead in solution at this stage contains about 34 % base (Table III, Solution 12, cols. V and VI). The p_H at this stage would be about 7.0 for 0.005 M solutions. With large excesses of reagent, the acidity approaches that of the reagent itself, about p_H 8.0 for 0.005 M dibasic lead acetate.

The behaviour of basic lead acetate towards neutralised solutions. In neutralised solutions the release of alkali (discussed on p. 464) which occurs when basic lead acetate is added to a salt which reacts to form an insoluble lead salt, will continue until an equivalent of lead has been added. On further addition of lead there will be reabsorption of alkali by the buffer action of the acetate fraction of the reagent, until finally the acidity will increase to that of the reagent itself. Under these conditions precipitation occurs during a phase of high alkalinity, and the precipitating reagent is therefore basic lead acetate and alkali, a mixture which has been used for the precipitation of almost all the sugars. The greater release of alkali, and the smaller and slower buffering action of acetate, when reagents of high basicity are employed, probably accounts for the widespread distrust of these reagents.

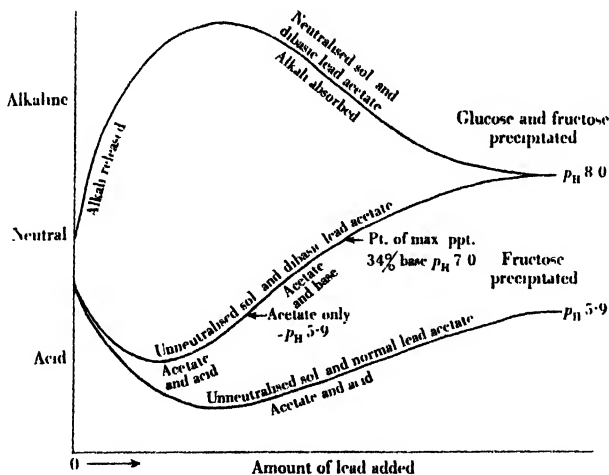


Fig. 1. Diagram to contrast the behaviour of neutralised and unneutralised solutions towards basic lead acetate.

After an alkali titration of plant extracts, using litmus or phenolphthalein to determine the end-point, many feebly acidic substances, such as phenols, which can react with lead, remain unneutralised. As a result the amount of alkali liberated on clarification of a plant extract, neutral to these indicators, is intermediate between that liberated from a completely neutralised solution and that from an unneutralised solution, and the danger of glucose loss is lessened.

Since the degree of neutralisation is variable (being greater in extracts with high proportions of carboxylic acids) the use of neutralised extracts cannot be recommended.

A simple schematic representation of the changes described is given in Fig. 1, where a curve for normal lead acetate is included for comparison. In unneutralised solutions the intermediate phase of high alkalinity is absent, and the point of maximum precipitation is approached from the acid side. The presence of salts of potassium and calcium *etc.* might be expected to give rise to free base on the addition of the lead reagent. There appears, however, to be sufficient acid remaining to buffer any such reaction with the plant salts.

The literature of sugar loss with regard to neutralisation

It has apparently not been generally realised that the frequently recorded losses of glucose, in aqueous solution, are due to neutralisation of extracts before precipitation and do not occur when this is omitted. Considered from the aspect of degree of neutralisation the literature of sugar loss becomes clear. Bryan [1908] added basic lead acetate to neutral sugar solutions containing magnesium sulphate and ammonium citrate, and as might be expected there was loss of both glucose and fructose. Similarly Deerr [1916] used neutral solutions containing invert sugar and sodium sulphate, and he recorded losses of over 50 % of the sugar, so that it is clear that both glucose and fructose must have been involved. Englis and Tsang [1922] added various salts to sugar solutions containing basic lead acetate. With neutral salts, sodium carbonate and sulphate, potassium oxalate and sodium potassium tartrate, losses of both glucose and fructose occurred. With disodium hydrogen phosphate neither sugar was lost. Since the standard basic lead acetate contained, presumably, 31–32 % base, and one-third of the phosphoric acid remains unneutralised, at the point of complete precipitation the solution would be slightly acid, and loss of sugar would not be expected. Their result would thus seem to depend on the use of a lead acetate of low basicity. With tannic acid, as might be expected, neither sugar was lost, an observation also made by Parkin [1912]. In every instance loss of glucose occurred only when neutral salts were present, and the recorded evidence does not therefore contradict the demonstration that glucose was not lost during the clarification of the unneutralised mangold extract.

The effect of clarification on glucose.

It appears to be established that the formation of a lead-sugar compound is a necessary preliminary to sugar loss in the presence of acids or salts which are capable of giving an insoluble lead precipitate. The lead-sugar compound, which, by itself, is fairly soluble, is carried down by this precipitate, presumably in a manner analogous to the removal of soluble lead acetate as a complex insoluble salt. To demonstrate, therefore, that no sugar is lost under given conditions of clarification, it is necessary only to show the absence of a lead-sugar compound.

In the clarification of Extract I with dibasic lead acetate the solution, at the point of maximum precipitation, which is approached from the acid side, contained 34 % base (Table III, Solution 12, cols. V and VI). This may be taken as the most basic condition to which the glucose was subjected. Accordingly the specific rotation of a 2 % solution of glucose in the presence of a 1 % lead acetate solution containing 34 % of base was determined. The observed specific rotation was $[\alpha]_D^{20} + 50.5^\circ$, the corresponding figure without lead being $+ 51.9^\circ$. The effect of lead, although definite, was therefore small, and it may be concluded that no appreciable amount of lead compound was formed during clarification

under the conditions described. This conclusion is based on the assumption that a large change of rotation occurs when lead is introduced into the sugar molecule. Fructose, which readily forms a lead compound, is changed from strongly laevorotary to dextrorotary by the addition of basic lead acetate [Pellet, 1896; Browne, 1912]. Sucrose behaves in a similar manner to glucose [Bates and Blake, 1907].

Allowance for losses of fructose during clarification.

Leaf extracts contain substances which are precipitated only under conditions of such low acidity that some loss of fructose is inevitable if lead clarification is to be efficient. The amount lost will be greatest when extracts of high concentration are used and the amount of sugar relative to other substances is low. Under such conditions 80% or more of the fructose may be precipitated. The error due to this loss of fructose may exceed that due to interference by substances other than sugar, and the use of basic lead acetate without allowance for losses of fructose is therefore inadvisable. In the mangold a correct estimate of fructose may be obtained by a determination, using a copper reagent, in the unclarified solution. Fructose can then be again estimated in the cleared extract in order to determine the true value for glucose by difference.

Sodium phosphate as a de-leading agent.

Disodium hydrogen phosphate is a satisfactory de-leading agent provided that a few drops of normal acetic acid are added to neutralise the solution to litmus, after the removal of the lead precipitate. Hydrogen sulphide was used in the experiments recorded here only because lead sulphide was required for analysis. After neutralising, a slight excess of phosphate is added and the precipitate removed either by centrifuging or by filtering. The filtrate is usually distinctly acid and should be again neutralised before use. Since dibasic lead acetate leaves only a very small proportion of lead in solution, there is no difficulty in keeping the quantity of phosphate in the cleared solution within the limits which may safely be present when the copper reagent is used.

THE CLARIFICATION WITH CHARCOAL OF EXTRACT I.

Extract I was cleared both by the application of charcoal alone and by the combined use of charcoal and lead. For the charcoal treatment the activated preparation "Suchar"¹ was added to the solution to be cleared and the whole shaken at room temperature for an hour, after which the charcoal was removed by filtering through a dry filter and sugar determinations were carried out. The results are shown in Table IV, in mg. of sugar per ml. of Extract I. The volume in ml. to which 1 ml. of the extract was diluted before treatment is again recorded.

Charcoal treatment decreased the estimates of both sucrose and fructose (see Solutions 13 and 14, Table IV). The decrease of sucrose must be attributed to loss of sugar, since it has already been deduced, from the results obtained from lead clarification, that substances other than sugar do not interfere with the determination of sucrose. It is of interest that the loss of sucrose is less during the first treatment with charcoal than during subsequent treatments; this is probably due to the competitive adsorption of other substances during the first treatment. From the figures of Archbold and Widdowson [1931] it can be calculated that the losses of fructose, when "Suchar" is used, are almost half

¹ Supplied by R. G. W. Farnell, Conduit Road, Plumstead, London, S.E. 18.

Table IV. *The estimates of sugar during the clarification of Extract I by charcoal or charcoal and lead.*

All weights are in mg. per ml. of extract.

Solution	Treatment	Colour	Apparent fructose		Apparent glucose			Apparent sucrose Copper
			Copper	Ferricyanide	Copper	Ferricyanide	Hypoiodite	
Uncleared	None. Figures are averages taken from Table I	Dark	65.5	75.8	11.0	50.4	77.7	34.4
13	One shaking with 150 mg. "Suchar" (vol. of dil. = 10)	Still deep	65.1	65.2	11.3	42.8	48.8	32.3
14	Two shakings with 150 mg. "Suchar" (vol. of dil. = 10)	Colourless	63.0	—	11.0	—	45.5	27.7
15	Three shakings with 150 mg. "Suchar" (vol. of dil. = 10)	"	—	—	—	—	44.6	—
13 A	As for 13, followed by normal lead acetate (118 mg. Pb; vol. of dil. = 12.5)	"	65.0	65.3	11.0	42.8	46.3	32.5
12	Dibasic lead acetate. Reproduced from Table I	Faint yellow	48.4	49.1	39.9	49.8	44.9	34.6
12 A	Solution 12, shaken with 13 mg. "Suchar" (vol. of dil. = 25)	Colourless	—	—	—	—	44.4	—
12 B	As above, but 40 mg. "Suchar" used	"	48.4	48.9	39.8	41.0	42.9	—
12 C	As above, but 160 mg. "Suchar" used	"	—	—	—	—	42.1	—

those of sucrose. On this basis the lowering of the estimate of fructose by the copper reagent, in Solutions 13 and 14 (Table IV), can also be accounted for entirely by the adsorption of sugar. At the same time it will be seen that the treatment with charcoal has reduced the difference between the copper and the ferricyanide estimates of fructose to negligible proportions (Solutions 13 and 13 A). It may therefore be concluded that, within reasonable limits of error, the copper estimate of fructose is not affected by the presence of other substances, and any error due to such substances falls entirely on the estimate of glucose. The correct estimate of fructose in Extract I is therefore that obtained from the unclarified solution (*i.e.* 65.5 mg. per ml. of extract).

After removal of the bulk of the interfering substances with lead, three solutions were further treated with charcoal in varying amounts. The results of sugar estimations on these solutions are shown in Table IV, Solutions 12 A, 12 B and 12 C. In Solution 12 B the combined treatment with lead and charcoal has brought the glucose estimations by copper and by hypoiodite into close agreement. This must mean that interfering substances have been almost entirely removed, and the copper estimate, which is almost exactly that obtained by the use of lead alone, is nearly a true one. Glucose may therefore be determined within reasonable limits of error after a suitable treatment with dibasic lead acetate alone.

THE ANALYSIS OF EXTRACTS II AND III.

The results obtained after treatment of Extracts II and III with lead acetate and charcoal are shown in Table V. As compared with Extract I the loss of fructose on clarification is larger, and there is greater relative error on the glucose estimate due to interference. Extract III contains a large amount of interfering substances relative to fructose, and in the unclarified extract (III) the fructose estimate by ferricyanide is more than double that by copper.

Table V. *The estimates of sugar during the clarification of Extracts II and III.*

Results expressed in mg. per ml. of extract. V is the volume to which 1 ml. of extract was diluted before each determination.

Treatment	Apparent fructose		Apparent glucose			Apparent reducing sugar, Ferri-cyanide	Apparent sucrose, Copper
	Copper	Ferri-cyanide	Copper	Ferri-cyanide	Hypoiodite		
Extract II. Unclearified	5.11	-	4.50	-	16.2	15.3	-
Dibasic lead acetate 55.2 mg. Pb; V, 3.3	1.30	-	3.07	-	4.01	4.77	2.87
Dibasic lead acetate 55.2 mg. Pb followed by "Suchar" 7 mg.; V, 5.2	1.30	—	3.06	-	3.42	-	-
Extract III. Unclearified	2.88	6.07	3.09	4.83	13.6	-	-
"Suchar" 60 mg.; V, 5	2.89	2.99	1.94	2.18	3.47	-	-
Normal lead acetate 50 mg. Pb; V, 2.5	2.90	-	2.47	—	9.89	-	-
Dibasic lead acetate 53.8 mg. Pb; V, 3.3	0.74	-	1.74	—	2.67	-	1.16
Dibasic lead acetate 53.8 mg. Pb followed by "Suchar" 8 mg.; V, 6.3	0.72	-	1.70	-	2.09	-	-

Treatment with "Suchar" brings the two estimates into close agreement, without appreciably altering the copper value. The difference between the copper estimates of glucose in the cleared and unclear solutions (Table V, Extract III) is 1.35 mg. (3.09-1.74), whilst the experimental error in the determination of fructose is only 0.03 mg., a negligible amount compared with the error of the glucose estimation in unclear solution. Thus in this case also the copper reagent can be used to estimate fructose in unclear solution, the errors in the estimation of sugars by copper falling entirely on the glucose fraction (*cf.* Extract I).

The copper estimate of glucose in Extract III after clarification with dibasic lead acetate (1.74 mg.) is again lowered by subsequent treatment with charcoal whilst the estimate by hypiodite remains considerably higher than the copper estimate. The value obtained after the lead treatment is thus probably only a good approximation to the true value of glucose in this case. When the amount of interfering substances is low relative to sugar the values for glucose obtained after lead clarification are probably within the limits of accuracy of the volumetric method employed, but if the relative amount of interfering substances is high the error may increase to a significant value. In these special circumstances further clarification may be necessary.

THE OXIDATION OF INTERFERING SUBSTANCES BY THE VARIOUS SUGAR REAGENTS.

It has already been pointed out that the reagents used to estimate sugar vary in the degree of oxidation which they effect. Thus by calculation from the usual thiosulphate factors it is found that, at a concentration of 20 mg. per 100 ml., 1 mg. of glucose absorbs 0.33 mg. of oxygen from the Harding and Downs copper reagent, 0.36 mg. from the modified copper reagent, 0.24 mg. from the ferricyanide reagent and 0.089 mg. from the hypiodite reagent. For 1 mg. of fructose the corresponding figures are 0.34, 0.35, 0.23 and 0 mg. respectively.

Assuming that the correct value for fructose is obtained by the application of the copper reagent to the unclarified extract and the correct value for glucose by the application of this reagent after suitable treatment with dibasic lead acetate, it is possible to calculate the amount of oxygen absorbed by the interfering substances in the unclarified extracts. For example, the estimate of glucose by the hypiodite reagent in the unclarified Extract I was 77.7 mg. per ml., the correct value being 39.9 mg. (see Table III). The oxygen absorbed by the interfering substances was therefore $(77.7 - 39.9) \times 0.089$ or 3.36 mg. per ml.

Calculations for all three reagents have been made in this way and are recorded in Table VI.

Table VI. *The amount of oxygen absorbed from the sugar reagents by interfering substances in the unclarified extracts.*

Extract	Oxygen absorbed, mg. per ml. of extract			Oxygen ratios		
	Copper	Ferri- cyanide	Hypo- iodite	Hypo- iodite Copper	Hypo- iodite Ferri- cyanide	Ferri- cyanide Copper
I	1.35	4.89	3.36	2.49	0.69	3.62
II	0.52	1.68	1.17	2.27	0.70	3.26
III	0.49	1.48	1.06	2.18	0.72	3.05
			Mean	2.31	0.70	3.31

According to the method used here, fructose was estimated by copper or ferricyanide after the oxidation of glucose with hypiodite. Substances other than sugar are subjected to oxidation at the same time as the glucose, and it follows that they will interfere with the estimate of fructose only if they retain some of their reducing powers after treatment with hypiodite. The data in Table VI show that the oxidising action of ferricyanide on substances other than sugar is stronger than that of hypiodite, and therefore interference with the estimate of fructose by ferricyanide may be expected, and is in fact found to occur. With copper the reverse is true, hypiodite being by far the stronger oxidising agent. These observations cannot by themselves be construed as proof that estimates of fructose by copper are free from interference but it at least explains the experimental fact that this is so.

A suggestion for the approximate estimation by an empirical correction factor of the error due to interference.

Although the three extracts were very dissimilar in their source and general properties, Table VI shows that the ratio of the oxygen absorbed from any two reagents by the interfering substances in them was reasonably constant. This means that the ratio of the errors of a sugar estimation by any two reagents remains reasonably constant for different extracts. By a comparison of the estimates for sugar by two reagents, it should therefore be possible to calculate the true sugar content.

It will be seen from the data in Table VI that the oxidising action of hypiodite on interfering substances is, on the average, 2.31 times as great as that of copper; further, during oxidation, 1 mg. of glucose absorbs 0.089 mg. of oxygen from hypiodite and 0.33 mg. from the unmodified Harding and Down's copper reagent. The error due to interference in the estimate of glucose will therefore be, on an average, $2.31 \times \frac{0.33}{0.089}$, or 8.6 times as great by hypiodite as by the copper reagent. To find the true value of glucose one could therefore estimate the

apparent glucose in unclarified solutions by the two reagents, divide the difference between the estimates by 7.6 and subtract the quotient from the copper estimate. For example, with Extract I, hypiodite and copper estimates of glucose in unclarified solutions were 77.7 and 44.0 mg. per ml. respectively (*v.* Table III). The difference between these estimates, *i.e.* 33.7 mg. per ml., divided by 7.6 is 4.4 mg. per ml., which may be taken as the error on the copper estimate. The corrected value for glucose will therefore be 44.0-4.4, or 39.6 mg. per ml. This compares with a value of 39.9 mg. per ml. estimated by clarification with dibasic lead acetate (Table III, Solution 12).

For the accurate use of such a correction factor it is necessary that the ratio of the oxygen absorbed by the interfering substances from hypiodite and copper should remain reasonably constant over the range of variation of the samples to be analysed. For different plants or different organs of the same plant different factors would probably be necessary, and it would also be essential to standardise the analytical procedure very carefully, particularly in the case of estimates by hypiodite in unclarified solutions where the period and temperature of oxidation adopted may cause considerable variation in the results.

Allowance for interference by the treatment with yeast of unclarified and clarified solutions.

Much attention has recently been paid to the possibility of using yeast to determine the error due to interference by substances other than sugar. The accuracy of such a procedure depends chiefly on the absence of interfering reactions between yeast and such substances. Lehmann [1931] showed that the reducing power towards ferricyanide of certain glucosides was increased by treatment with yeast and pointed out that reactions of this type would cause an underestimation of sugar. Kerstan [1934], on the other hand, found that estimates of sugar by ferricyanide were lower if extracts were first partially clarified with charcoal, and it would therefore seem that reactions may occur which tend to overestimation of sugar. Kerstan's results may be due to the removal of substances other than sugar by yeast, a possibility which is supported by Yemm's [1935] observation that yeast partly removes the colouring matter from extracts of starved barley leaves. There are therefore sources of uncertainty as to the accuracy of the results obtained, and no evidence exists to show what the magnitude of the error is likely to be. Estimations were therefore carried out on the three mangold extracts so that results could be compared with those obtained by the use of dibasic lead acetate. The clarified and unclarified solutions were diluted until they contained a maximum of 50 mg. of sugar (glucose, fructose and sucrose) per 100 ml. and were then mixed with an equal volume of a 10% suspension of washed baker's yeast. After standing at 35° with occasional stirring, the yeast was removed by centrifuging and the liquid analysed. Allowance was made for the reducing power of substances introduced into the solution by yeast. The results of estimations by the use of both copper and hypiodite reagents are shown in Table VII.

The first row of figures gives the estimates for glucose by the copper and hypiodite reagents in the unclarified solution, and the fourth row the estimates of glucose by the copper reagent after clarification with dibasic lead acetate. These figures are reproduced from Tables III and V.

The third row of Table VII shows the estimates of glucose as the differences between the values obtained before and after treatment with yeast. With the hypiodite reagent these values are even higher than the uncorrected estimates obtained by the copper reagent in unclarified solution. This high value is

Table VII. *The estimation of glucose as the difference in reducing powers of clarified and unclarified extracts before and after treatment with yeast.*

All results expressed as mg. of glucose per ml. of extract.

	Extract					
	I		II		III	
	Copper	Hypo-iodite	Copper	Hypo-iodite	Copper	Hypo-iodite
Unclarified						
Before treatment	44.0	77.7	4.50	16.2	3.09	13.6
After treatment	1.6	32.3	1.64	11.5	1.53	10.6
Difference	39.4	45.5	2.86	4.7	1.56	3.0
Clarified						
Before treatment	39.9	—	3.07	—	1.74	—
After treatment	0.8	—	0.17	—	0.18	—
Difference	39.1	—	2.90	—	1.56	—

possibly similar to that found by Kerstan with ferricyanide, and the use of yeast does not obviate the need for a careful choice of sugar reagents. With the copper reagent the estimates of glucose obtained by the use of yeast agree closely with those obtained by using dibasic lead acetate.

In obtaining these copper values, use has been made of the observation that with copper the error due to interfering substances falls quantitatively on the estimate of glucose. The whole reducing power remaining after treatment with yeast has therefore been calculated in terms of glucose. In the clarified solution the estimates of glucose by copper are again very nearly equal to the corresponding estimates in unclarified solution, the residual reducing power after fermentation being much reduced by the lead treatment.

With the copper reagent lead clarification makes little difference to the estimate of glucose obtained after yeast treatment. For the analysis of mangold extracts therefore the fermentation method appears to be highly accurate, probably giving even more trustworthy results than those obtained after lead clarification. Any reactions which may occur between yeast and substances other than sugar do not appear to affect the copper reagents. This result may of course be due to the counterbalancing of opposing errors, rather than to the absence of any interfering reactions at all.

When the ferricyanide reagent is used the errors of estimation are shared between the glucose and fructose fractions, and in consequence yeast was only used to estimate reducing sugar as a whole. For Extract I the following figures were obtained: Estimate of reducing sugars in the unclarified solution (from Table III) 126.2 mg. per ml., after treatment with yeast 18.6 mg. per ml., difference 107.6 mg. per ml. With the copper reagent the value was 105.4 mg per ml. (fructose 65.5 mg., glucose 39.9 mg.). Ferricyanide therefore slightly overestimates sugar when used in conjunction with yeast.

DISCUSSION.

When concentrated, unneutralised extracts are clarified with the correct quantity of dibasic lead acetate trustworthy estimates of glucose can be obtained by the use of copper reagents. The clarification can be shown to be efficient by comparing the estimates obtained by the copper and hypiodite reagents, and at the same time it can be shown by polarimetric observations that no appreciable losses of glucose occur during the process. This method may therefore be used to

provide a standard estimate of glucose in the mangold extracts. Sucrose is also unaffected by basic lead acetate, but in the case of fructose efficient clarification cannot be effected under conditions which preclude losses of this sugar. To obtain information about fructose careful use of charcoal was necessary. Estimates of fructose are, however, free from interference if the copper reagent is used, and no clarification is necessary.

For routine analysis clarification with dibasic lead acetate is unsatisfactory, since concentrated extracts are required, the method is rather tedious, and fructose must be determined in both cleared and uncleared solutions to allow for the sugar lost.

Although charcoal may profitably be used as a clarification agent in special circumstances, generally speaking it is less suitable than lead. There is no means of determining the amount to use, whereas with dibasic lead acetate the amount can be found by trial. Whilst fructose alone is lost during lead clarification charcoal will cause losses of both fructose and sucrose if it is used in amounts sufficient for adequate clarification: in both respects therefore charcoal is an inferior reagent.

The fermentation method is well suited to the needs of routine estimations of sugars in the mangold leaf if it is used in conjunction with a copper reagent. It is very accurate and rapid and may be used with or without the separate estimation of fructose and glucose. Nevertheless, it should not be regarded as a standard method since, in spite of its accuracy when applied to mangold leaves, it lacks the trustworthiness which is associated with the method of lead clarification. In the fermentation method complications may be introduced by the enzymes present and there is no means of ascertaining what reactions are occurring between yeast and substances other than sugar: whilst with dibasic lead acetate the degree of clarification can be determined by comparing the estimates of glucose by copper and hypiodite. In view of the elements of uncertainty it is considered that the application of the fermentation method to the routine estimation of sugars in any plant should be preceded by a comparison of results with those obtained by the use of dibasic lead acetate.

With regard to sugar reagents there can be little doubt as to the superiority of copper reagents. Ferrieyanide reagents are far more sensitive to the presence of substances other than sugar and fail to provide a method for fructose which is free from interference. It has also been observed that they are very active towards solutions of invertase and towards those substances which are introduced into solution by yeast. No important compensating advantages have yet been discovered for ferrieyanide reagents and their use cannot be recommended.

III. NOTE ON THE APPLICATION OF COPPER REAGENTS TO UNCLARIFIED EXTRACTS.

In the application of copper reagents to the estimation of reducing sugars in plant extracts, errors of estimation may arise which are in no way connected with the reducing power of substances other than sugar which may be present. If efficient methods of clarification are used, all types of error are removed simultaneously and there is no need to distinguish between them. Recently however there has been a tendency to replace clarification methods by fermentation methods, using unclarified solutions.

In unclarified solutions two types of error arising from sources other than the reducing power of substances besides sugar have been observed. Both types are most likely to occur in extracts which are poor in sugar but rich in other substances. Thus reducing sugar may be underestimated owing to the presence of substances which protect the copper salts from complete reaction with the iodine. Errors of this type are most common in cold weather and may be overcome either by shortening the time of cooling the tubes prior to the addition of the oxalate/iodide solution, and by keeping both this solution and the sulphuric acid solution in a warm place, or by allowing the tubes to stand for at least 15 min. after liberating the iodine. Extracts prone to this type of error should be analysed only after considerable dilution: this is the easiest way of overcoming the difficulty. When interference of this sort is present, repeated titrations invariably disagree, and it is therefore unlikely that the error can be overlooked.

Some constituents of extracts have the power of altering the oxidising activity of the reagent towards sugar. This error may be detected by adding a known quantity of sugar to the extract; if interference is present the recovery of this sugar is not quantitative. Alternatively the extract may be re-analysed after dilution: if interference is present the amount of estimated sugar is not proportional to the concentration. This error may also be removed by diluting the extracts. With extracts of fresh mangold leaves appreciable errors seem unlikely if the reducing sugar concentration is kept below 10 mg. per 100 ml.

Attention may be drawn to the fact that it is convenient and more accurate to reduce the amount of iodate in the copper reagent if dilute extracts are to be analysed. The strength of the thiosulphate may also be suitably reduced. No alteration of the sugar factor is necessary. The matter has been discussed by Shaffer and Somogyi [1933].

IV. TESTS FOR THE PRESENCE OF RAFFINOSE, MALTOSE, GALACTOSE AND PENTOSE.

Little is known about the presence in leaves of sugars other than glucose, fructose and sucrose. Davis *et al.* [1916] examined the mangold leaf for pentose and maltose, and they record the presence of pentose in appreciable amounts but no maltose. They based their estimations on the selective action of pure strains of yeast towards the various sugars, using relatively concentrated extracts and allowing fermentation to proceed for several weeks. Since 1916, methods have been devised for the separation of sugars in blood, urine *etc.*, which involve the rapid removal of sugars from very dilute solution by relatively large amounts of yeast [Harding and Nicholson, 1933]. This newer technique allows estimations to be conducted under easily controlled conditions and is superior in every way to that used by Davis *et al.* It was therefore decided to re-investigate the sugars of the mangold leaf, and tests were made, not only for maltose and pentoses, but also for raffinose and galactose.

Method. Samples of yeast were suspended in water and washed by repeated centrifuging. The sugar solution to be treated was then added to a weighed quantity of washed yeast in a centrifuge-tube, and the yeast was distributed by stirring with a glass rod. After the reaction the yeast was removed by centrifuging, and samples of the liquid were taken for analysis by the modified Harding

and Downs copper reagent (see p. 458). Allowance was made for those substances, having reducing power, which are introduced into the solution by the yeast; under the conditions used, this allowance was generally equivalent to only 0.01–0.02 ml. of 0.01 *N* thiosulphate.

It is of interest that, judging from trials with the Hanes [1929] modification of the Hagedorn-Jensen method, ferrieyanide reagents appear totally unsuited to this type of work. They are about six to ten times as reactive as the copper reagent towards substances introduced into the solution by yeast.

The three extracts of mangold leaves already discussed in Part II were used. Before treatment with yeast they were clarified in concentrated, unneutralised solution by dibasic lead acetate and decolourised with sodium phosphate. The results are summarised in Table VIII in which are reproduced, for the purpose of comparison, the estimates of sucrose, fructose and glucose obtained by chemical methods.

All samples of yeast were tested against the various sugars to ensure that they had the activity or inactivity required of them.

Table VIII. *Estimates of various sugars in mangold leaf extracts.*

Sugar	Extract		
	I	II	III
Sucrose	34.4	2.87	1.16
Fructose	65.5	5.11	2.88
Glucose	39.9	3.07	1.74
Raffinose	< 1.0	< 0.09	< 0.08
Maltose	< 0.6	< 0.06	—
Galactose	< 2.8	< 0.22	< 0.10
Pentose	0.0	< 0.04	—

Raffinose. Raffinose has not been isolated from the mangold, although it is a recognised constituent of the closely related sugar beet. Raffinose may be determined by treatment first with a top yeast, which produces melibiose, and then estimation of the loss of reducing power on subsequent treatment with brewer's bottom yeast.

25 ml. of a solution containing 6–9 mg. of total sugar were treated with 1.5 g. of baker's yeast for 60 min. at 35°. After removing the yeast and withdrawing samples for analysis, the remainder (15 ml.) was treated with 1.0 g. of brewer's bottom yeast for 120 min. at 35° to remove melibiose. No significant change in reducing power was observed in any of the extracts subjected to this treatment, and it was therefore concluded that raffinose, if present, occurs only in amounts too small for detection by the method used. The figures recorded in Table VIII show the maximum amount which could have escaped detection.

Maltose. 25 ml. of a solution containing 6–8 mg. of total sugar were treated with 1.5 g. of a maltase-free *S. marxianus* for 30 min. at 38°. After removing the yeast and withdrawing samples for analysis the remainder (15 ml.) was treated with 0.5 g. of baker's yeast for 30 min. to remove maltose. No significant change in reducing power was observed. The result is recorded in Table VIII, and confirms that of Davis *et al.* [1916].

Galactose. The method used was that of Harding and Grant [1931] who employed a galactose-trained yeast to remove galactose subsequent to the removal of glucose. Baker's yeast is feebly reactive towards galactose, and in the presence of fructose and sucrose the method gives only approximate results,

since these sugars react more slowly than glucose, and the galactose is exposed for a considerable period to the action of the yeast.

25 ml. of a solution containing 6–9 mg. of total sugar were treated with 1.5 g. of baker's yeast for 30 min. at 38°. After removal of the yeast and of samples for analysis, the remainder (15 ml.) was treated for 30 min. at 38° with 0.04 g. of galactose-trained yeast. No significant change of reducing power was observed. Under the conditions used, the baker's yeast was able to remove 0.21 mg. of galactose, so that the amount of galactose which might remain undetected was rather high.

Pentoses. According to Davis *et al.* [1916], the mangold leaf contains relatively large quantities of pentose, but no confirmation of this result could be obtained. Pentoses are determined either by the reducing power of the non-fermentable residue after clarification or by the yield of furfuraldehyde obtained by distillation with acid. Both methods require a high degree of clarification if accurate results are to be obtained. The agreement between the two methods found by Davis and Sawyer [1914] can only be regarded as fortuitous since experience with the method of lead clarification used by them shows that their estimates for pentose might readily be accounted for by imperfect clarification.

Substances other than sugar also affect the furfuraldehyde method. In order to demonstrate this, a sample of Extract I was treated with dibasic lead acetate in amount sufficient for maximum precipitation. The supernatant liquid was removed after centrifuging and the precipitate then treated with dilute sulphuric acid equivalent in amount to the lead base which had been added. After stirring and allowing the acid to react, dibasic lead acetate was again added in amount to give maximum precipitation, and the precipitate was separated. Owing to the accumulation of acetates, this second precipitation was far from complete, and no sugar was carried down, analysis showing that even fructose was absent. The precipitate was then distilled with hydrochloric acid (sp. gr. 1.06) and the distillate, when treated with phloroglucinol and aniline acetate was found to contain furfuraldehyde, which must have been derived from substances other than sugar.

To obtain confirmation of the absence of pentose use was made of a double clarification. Solutions cleared with dibasic lead acetate were diluted, and 25 ml. containing about 15 mg. of total sugar were shaken in the cold with 70 mg. of charcoal (Suchar) for an hour. This treatment was found scarcely to affect pentose. After removing the charcoal, the solutions were treated with baker's yeast, the reducing power of the unfermentable residue was small and insignificant. The value is entered in Table VIII.

SUMMARY.

Part I. The application of micro-reagents of the copper and of the ferri-cyanide groups to the analysis of mixtures of fructose, glucose and sucrose is discussed.

Methods are described whereby fructose may be estimated by either type of reagent after the oxidation of glucose by hypoiodite.

Chlorides affect micro-reagents, and hydrochloric acid is, therefore, generally unsuitable for the inversion of sucrose. If invertase is used for this purpose, precautions are necessary for accurate estimations by ferri-cyanide.

Full details are given of a modification of the Harding and Downs iodide-free copper reagent.

Part II. Estimates of glucose, fructose and sucrose by the methods described were made in clarified and unclarified extracts of mangold leaves.

In the unclarified extracts the estimates for sucrose, after hydrolysis with invertase, are free from interference by substances other than sugar with both copper and ferricyanide reagents. With the copper reagent the error due to the presence of these substances falls entirely on the estimate of glucose, but with the ferricyanide reagent the error is large and is divided between the estimates of fructose and glucose. Copper reagents were found to be distinctly superior to ferricyanide reagents.

Clarification with normal lead acetate is inefficient. With basic lead acetate the precipitate is a complex acetate, and its solubility increases with the content of acetate. To reduce its solubility, the acetate content should be reduced as far as possible by the use of dibasic lead acetate, but even with this reagent the precipitate is still appreciably soluble. Extracts to be clarified should therefore be concentrated, and methods which involve the washing of the precipitate should not be used. An excess of basic lead acetate should be avoided since it tends to redissolve the precipitate. Solutions may be delead with sodium phosphate after neutralisation with acetic acid.

Efficient clarification without the simultaneous loss of fructose is impossible. Allowance is made for this loss by estimating fructose with a copper reagent both before and after clarification.

To avoid losses of glucose, extracts should not be neutralised before clarification. A consideration of the behaviour of basic lead acetate towards neutralised and unneutralised solutions explains previous data of sugar loss recorded in the literature.

Charcoal is unsatisfactory as a general clarifying agent, but may be used with caution in special cases.

The use of yeast to allow for interference in unclarified extracts gives with a copper reagent an accurate and rapid method suitable for routine analyses. With ferricyanide or with hypiodite the method is inaccurate and overestimates sugar.

A suggestion is made for the use of an empirical correction factor to allow for interference.

Part III. If extracts are relatively rich in substances other than sugar they should be analysed only after considerable dilution.

Part IV. Tests were made for raffinose, maltose, galactose and pentoses by selective fermentation. If these sugars are present at all they occur in amounts which are too small for detection.

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LXXIV. THE OXIDATION OF AMINO-ACIDS BY HYPOCHLORITE.

I. GLYCINE.

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FROM the work of Langheld [1909] with hypochlorite and of Dakin [1916] with chloramine-T it is known that active chlorine may produce from an amino-acid either the aldehyde or cyanide with one less carbon atom together with carbon dioxide and (in the first instance) ammonia. Chlorination of the amino-group has been supposed to constitute the first stage of the reaction. Wright [1926] considered the nature of the reaction to depend on the relative proportions of active chlorine and amino-acid present; with excess of chlorine it was regarded as oxidation and with excess of amino-acid as chlorination.

In the work to be presented, the optimum conditions for the complete oxidation of glycine by hypochlorite have been determined and the course of the reaction studied quantitatively. The investigation arose out of an attempt to use a "chlorine demand" figure for the evaluation of sewage and certain trade wastes.

EXPERIMENTAL.

The reaction between glycine and chlorine was followed iodimetrically. The source of chlorine was the commercial hypochlorite solution of Messrs Laporte Ltd. (Luton), a typical analysis of which showed:

			%
Available chlorine	>15.0
Sodium chloride	13.4
Sodium hydroxide	0.77
Sodium carbonate	0.74

In all experiments, controls consisting of the hypochlorite solutions without glycine were run concurrently. All experiments were carried out at room temperature unless otherwise stated.

(a) Amount of chlorine used.

Six series of bottles, each series containing 14 mg. of available chlorine and different amounts of glycine ranging from 1 to 30 mg. in 100 ml. of water, were allowed to stand $\frac{1}{2}$, 1, 2, 3, 4 and 5 hours and the residual Cl titrated. The results for the 2, 3, 4 and 5 hour series were practically identical, indicating that the reaction under these conditions is complete within 2 hours. In Fig. 1 the percentage of available Cl remaining is plotted against mg. of glycine present for the $\frac{1}{2}$, 1 and 2 hour series. The curves are generally similar to those given by Wright [1926], who interpreted the downward portion of the curve as oxidation and the upward portion as mainly chlorination. As an alternative view it seemed possible that glycine in the upward part of the curve was reacting only very slowly.

Several series of oxidations were then run at various levels of glycine. The time allowed was, in every case, 2 hours. The mg. Cl added were plotted against mg. Cl used and a curve obtained for each level of glycine as given in Fig. 2. For 2 mg. of glycine it is seen that when 10 mg. of Cl are added, the curve

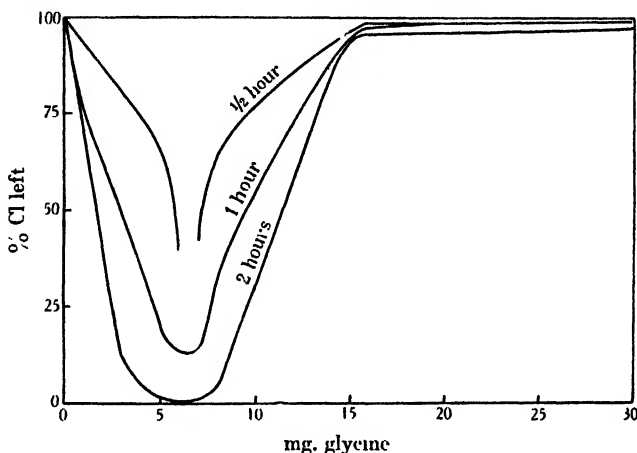


Fig. 1. Percentage of Cl used by glycine in various amounts.
14 mg. chlorine; glycine varied.

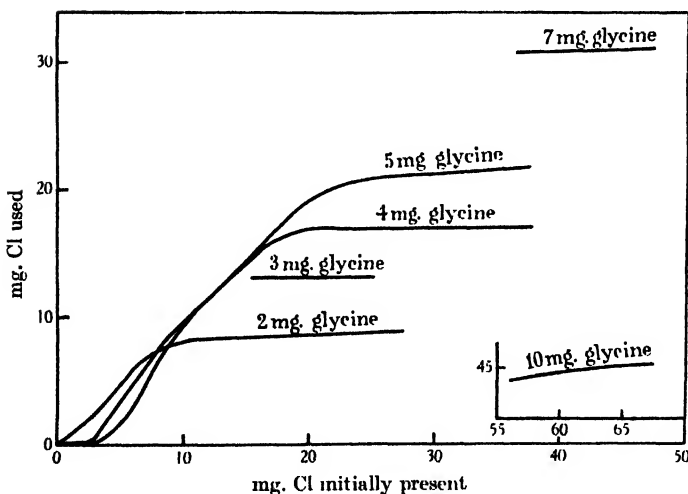


Fig. 2. Quantitative uptake of Cl by glycine.

levels off, indicating that 2 mg. of glycine react quantitatively with 8.55 mg. of Cl. In other words, the presence initially of at least 5 times as much Cl as glycine is necessary before the glycine will use a constant amount of chlorine. Examination of the other curves confirms this, and from them it was ascertained that 1 mg. of glycine uses 4.26 mg. of Cl.

If, for all the values obtained thus far in both experiments (exclusive of the $\frac{1}{2}$ and 1 hour series in Fig. 1 which cannot be considered to have come to com-

pletion), the ratio of Cl initially added to glycine be plotted against the percentage of Cl used, a general curve for the reaction is obtained and shown as the continuous line in Fig. 3.

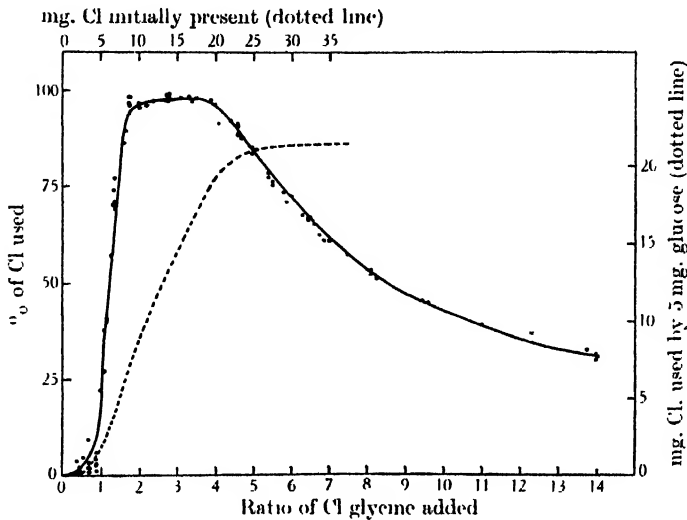


Fig. 3. Effect of Cl concentration.

Table I. *Determination of chlorides.*

A. Variation of Cl with 5 mg. of glycine.

Initial available Cl mg.	Ratio of initial Cl to glycine	Chloride-Cl			One half available Cl used mg.
		At start mg.	After 2 hours mg.	Increase mg.	
14.2 Contr.	—	9.25	9.25	—	—
14.2 Contr.	—	9.25	9.25	—	—
14.2	2.84	9.25	15.9	6.6	6.65
19.9	3.98	12.95	21.9	8.95	9.4
22.7	4.54	14.8	24.7	9.9	10.1
25.5	5.1	16.65	26.9	10.25	10.3
28.4	5.68	18.5	28.9	10.4	10.55
34.1	6.82	22.2	32.8	10.6	10.6
39.7	7.94	25.9	36.6	10.7	10.75

B. Variation of glycine with 14 mg. Cl.

Glycine mg.	Ratio of initial Cl to glycine	Chloride-Cl		One half available Cl used mg.
		After 2 hours mg.	Increase mg.	
Contr.	—	9.0	—	—
Contr.	—	9.0	—	—
1	14.0	11.2	2.2	2.15
3	4.66	15.0	6.0	6.15
4	3.5	15.4	6.4	6.75
5	2.8	15.6	6.6	6.75
7	2.0	15.3	6.3	6.65
8	1.75	14.2	5.2	6.15
9	1.55	13.6	4.6	5.5

C = Control containing no glycine.

(b) *Determination of chlorides.*

The hypochlorite solution employed contained alkali in the form of sodium hydroxide and sodium carbonate, together with a considerable amount of chloride. When the available Cl is determined by titration in the usual way, not only the Cl actually present in the NaOCl is titrated, but also an equivalent amount of Cl liberated from the chloride, the available Cl being twice the amount of Cl actually present as hypochlorite. If the reaction of Cl with glycine is considered ultimately to be one of oxidation, then for every two hypochlorite Cl atoms or for every four atoms of available Cl two mols. of chloride are formed. In other words, the increase of chloride should be equal to one-half the amount of available Cl used. To test this point, two series of experiments were made in duplicate one keeping the glycine constant and varying the Cl, the other keeping the Cl constant and varying the glycine. After 2 hours one bottle was titrated for available Cl and the other for chloride. In all cases in which, by reason of the relative proportions of Cl and glycine present, the oxidation was complete (figures in bold type), the increase in chloride agreed well with one half the available Cl used (Table I). When oxidation was incomplete, chloride production lagged behind Cl utilisation.

(c) *Factors affecting the rate of reaction.*

Physical. Three bottles containing 4 mg. of glycine per 100 ml. and approximately 20 mg. per 100 ml. of available Cl and three controls of hypochlorite alone were set up. One sample and one control were left at 15–17°, one set was placed in a dark cupboard at the same temperature and the other set in an incubator at 30°. 100 ml. were withdrawn from each bottle at various time intervals and titrated for available Cl. All three controls showed no loss of available Cl (Table II).

Table II. *Effect of heat and light on chlorine utilisation.*

Time hours	Incubator, 30° darkness mg. Cl used	Room temp. daylight mg. Cl used	Room temp. darkness mg. Cl used
0.25	0.6	—	0.7
0.5	2.4	1.9	2.2
0.75	5.2	—	4.4
1.0	11.6	6.65	7.15
1.25	16.6	—	13.6
1.5	16.95	15.6	14.8
1.75	17.2	—	15.7
2.0	17.2	16.5	16.4
4.0	—	17.0	17.0

Table III.* *Effect of 500 candle-power light on Cl utilisation.*

Time hours	500 c.p. light mg. Cl used	No light mg. Cl used
0.5	8.55	7.65
0.75	12.75	11.8
1.0	14.5	13.9
1.25	15.8	15.8
1.5	16.5	16.4
1.75	16.9	16.9
2.25	17.2	17.1

* The hypochlorite used in this experiment was from a fresh source, so that the figures are not directly comparable with those of Table II because of alkalinity conditions, which will be discussed later.

The effect of a 500 c.p. light at a distance of 1 ft. on Cl utilisation was tried. The bottle subjected to the light and its control were placed in a glass water-bath in order to eliminate the effect of heat. Another set for comparison was placed in a water-bath of the same temperature but not exposed to the light (Table III).

Chemical. Reaction with hypochlorous acid. The effect of employing free hypochlorous acid instead of sodium hypochlorite was tested. The acid was prepared by passing Cl_2 into a suspension of mercuric oxide and then distilling under reduced pressure. 2 mg. of glycine as the Na salt per 100 ml. water were used. It was found necessary to let the bottles stand longer than the usual 2 hours for completion (Table IV).

Table IV. *Amount of Cl as HOCl used by 2 mg. glycine.*

Cl added mg.	Chlorine used					
	2 hours mg.	5 hours mg.	1 day mg.	2 days mg.	3 days mg.	4 days mg.
3	2.1	2.3	2.35	2.35	2.5	2.45
6	3.6	4.75	5.45	5.6	5.7	5.6
9	4.0	5.0	6.65	7.4	7.6	7.6
15	3.9	5.0	7.05	8.25	9.1	9.05
21	3.05	4.9	7.14	8.4	9.05	9.15

The reaction proceeded very slowly so that 3 days were required. Slightly more Cl (9.05 mg.) was used up by 2 mg. of glycine than was found with sodium hypochlorite (8.55 mg.). The HOCl solution is, of course, acid and completely masks the effect of the small amount of alkali (0.0266 m.mol. per 100 ml.) used to convert glycine into its sodium salt.

Reaction with chlorine water. A similar experiment was carried out with chlorine water. The controls tended to lose Cl at rather varying rates, so at each time interval three control determinations were made and the loss of available chlorine computed from an average of the three (Table V).

Table V. *Amount of Cl used up from Cl water by 2 mg. glycine.*

Initial Cl mg.	Chlorine used				
	2 hours mg.	5 hours mg.	1 day mg.	2 days mg.	3 days mg.
5.5	2.3	3.9	-	-	-
10.95	3.05	4.4	-	-	-
16.4	2.9	4.4	7.1	8.2	8.6
21.9	3.2	4.6	7.3	8.6	8.5
27.4	3.1	5.2	8.1	8.8	8.6

The reaction with chlorine water proceeded slightly faster than with HOCl, the amount of Cl used (8.6 mg. per 2 mg. glycine) agreeing closely with that from sodium hypochlorite.

Effect of acid and alkali on the rate of reaction. The hypochlorite solution employed contained 0.88 % sodium hydroxide and 0.76 % carbonate, exactly 10 ml. of the strong solution being diluted to 500 ml. as the source of chlorine for the following experiments. Large bottles were set up containing 4 mg. of glycine and 10 ml. of the dilute NaOCl solution (containing 20 mg. available Cl) per 100 ml. The acidity of the glycine (4 mg. = 0.0532 m.mol. of acid) and the

alkalinity of the hypochlorite (10 ml. dilute solution = 0.0727 m.mol. of alkali) were taken into consideration and various amounts of H_2SO_4 or NaOH were added so that a range from 25 m.mol. of alkali to 1 m.mol. of acid per 100 ml. of reaction mixture was covered. A large number of controls containing similar additions of acid and alkali were run simultaneously, since in stronger concentrations of acid and alkali the available Cl decreases slowly on long standing. 100 ml. were withdrawn and titrated at various time intervals, the results being given in Figs. 4 and 5. In those cases in which oxidation was complete (Fig. 4) 17 mg. of available Cl were

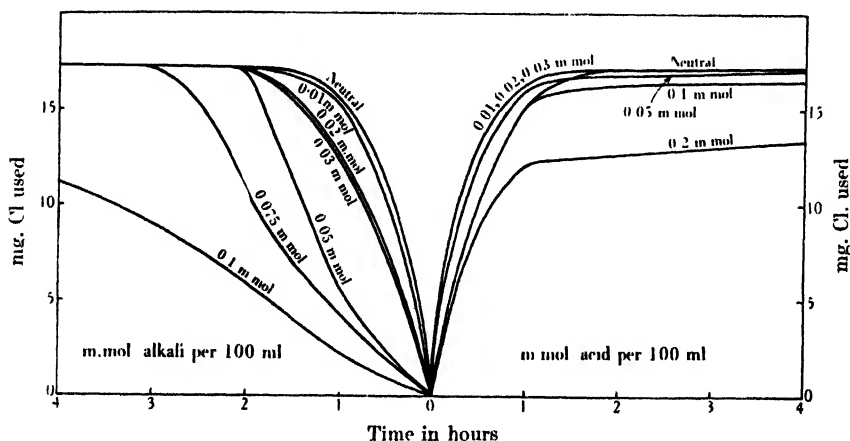


Fig. 4. Effect of acidity or alkalinity on rate of reaction.
4 mg. glycine present per 100 ml.

utilised by 4 mg. of glycine. As the alkali concentration increased, the rate of Cl utilisation decreased. On the acid side the 0.01–0.05 m.mol. reaction mixtures all used Cl at about the same rate, which was slightly faster than in the neutral¹ experiment. After the first hour the 0.1 m.mol. acid sample proceeded at a somewhat slower rate than the neutral one, and the 0.2 m.mol. sample was still slower, not having come to completion in 8 hours. A similar result was obtained with the 0.075 and 0.1 m.mol. alkaline reaction mixtures.

The curves in Fig. 5 represent concentrations of acid and alkali > 0.1 m.mol. per 100 ml. On the acid side, an increase in acid concentration again retarded the rate of reaction. The alkaline side is more complicated for, in contrast with the results in Fig. 4, the greater the amount of alkali the more Cl is used. It seems that small additions of alkali retard the reaction up to a certain point, after which further additions slowly increase the rate of reaction. A few more 2-hour determinations of the amount of available Cl used up were made around what seemed to be the critical point of 0.25 m.mol. of alkali per 100 ml. These, together with those already obtained, are given in Table VI.

This confirmed the fact that the point of reversal due to the effect of alkali was at 0.25 m.mol. of alkali. The slow reactions shown in Fig. 5 were followed for 20 days, but loss of Cl in the controls made interpretation difficult. In general it can be said that the rate of reaction was very slow after a few days in these comparatively strongly alkaline and acid mixtures.

¹ 10 ml. dilute NaOCl contain 0.0727 m.mol. alkali. 4 mg. glycine contains 0.0532 m.mol. acid. 0.0195 m.mol. acid was accordingly added per 100 ml. to neutralise the excess of alkali.

Table VI. *Additions of alkali to a mixture of chlorine and glycine.*

Cl concentration — 23 mg. per 100 ml. Glycine concentration — 4 mg. per 100 ml.

m.mol. alkali present per 100 ml.	mg. Cl used 2 hours
---	17.05
0.05	16.85
0.075	10.7
0.1	6.0
0.14	5.7
0.19	5.6
0.22	5.5
0.25	5.15
0.27	5.2
0.30	5.5
0.4	5.6
0.5	6.1
0.7	6.8
1.0	7.6

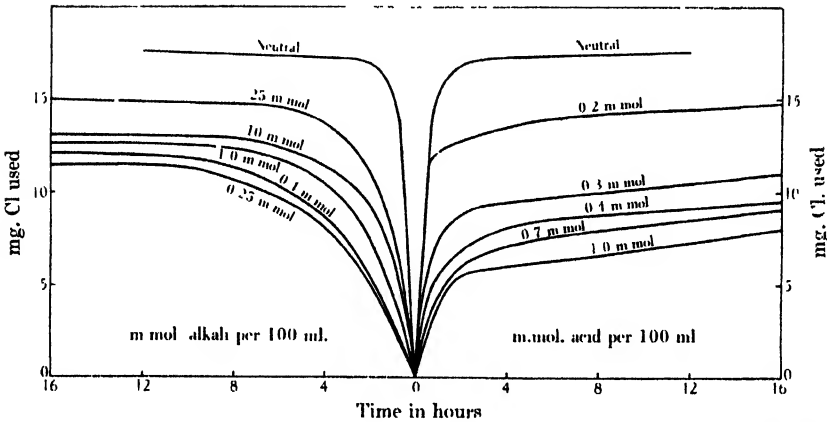


Fig. 5. Effect of acidity or alkalinity on rate of reaction. 4 mg. glycine present per 100 ml.

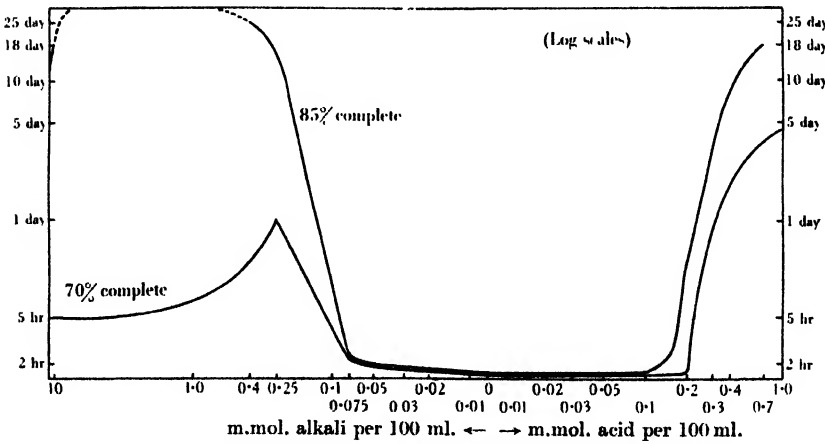


Fig. 6. General variation in rate of reaction.

The general variation in the rate due to the acid or alkali content is, perhaps, best shown in Fig. 6, where the time and the acid or alkali content are plotted on a logarithmic scale. The curves represent 70 and 85 % completion of the reaction, figures which have been arbitrarily chosen. Within the limits of 0.1 m.mol. acid and 0.1 m.mol. alkali per 100 ml. the reaction is rapid, outside these limits, slow, with a reversal on the alkaline side. A curve of a somewhat similar nature was given by Clibbens and Ridge [1927] for the hypochlorite oxidation of cotton.

As a result of these experiments which have shown that very small additions of acid or alkali may greatly affect the rate at which Cl is used by glycine, some former results may be better interpreted. The 2-hour curve in Fig. 1 is very similar to that given by Wright [1926] for 5 hours. Wright stated that he used an alkaline solution of glycine which no doubt retarded the reaction. Similarly the oxidation of glycine by chlorine water and by HOCl was slow, owing to the acidity of these solutions.

Hydrogen ion concentration.

The changes in p_H during the course of the reaction were followed by means of a glass electrode. Three samples, each containing 4 mg. of glycine and 10 ml. of dilute sodium hypochlorite solution (23.0 mg. Cl) per 100 ml. were set up. One received alkali to make the final concentration 0.168 m.mol. alkali per 100 ml., one received no addition of acid or alkali but had present 0.02 m.mol. of alkali per 100 ml. due to the alkali present in the hypochlorite solution, and the third received acid to make the final concentration 0.1 m.mol. acid per 100 ml. The changes in p_H and the Cl used were determined at various time

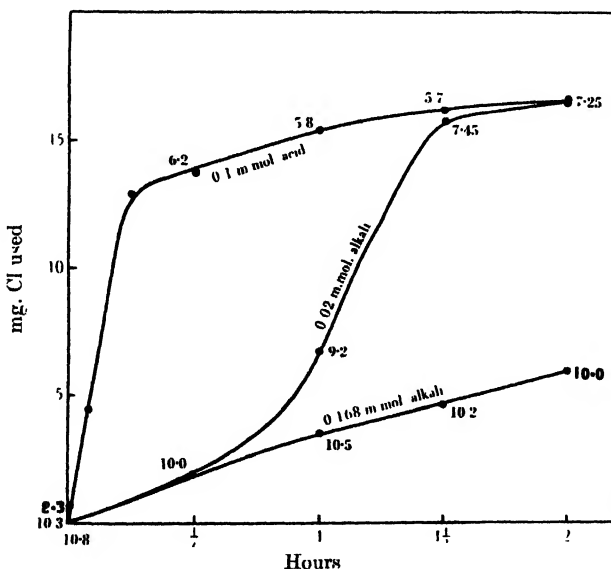


Fig. 7. Effect of p_H on Cl utilisation. Figures in bold type are p_H readings by glass electrode.

intervals. The data presented in Fig. 7 bring out the change in the rate of Cl utilisation due to differences in p_H . The process of oxidation of glycine produces a fall in p_H , the Cl uptake being most rapid between p_H 7.0 and 9.0. Reactions

which were proceeding slowly owing to a high initial p_H were greatly accelerated by suitable adjustment to neutrality (as calculated). Examples are given in Fig. 8, the dotted lines indicating the rate of the reaction if unadjusted.

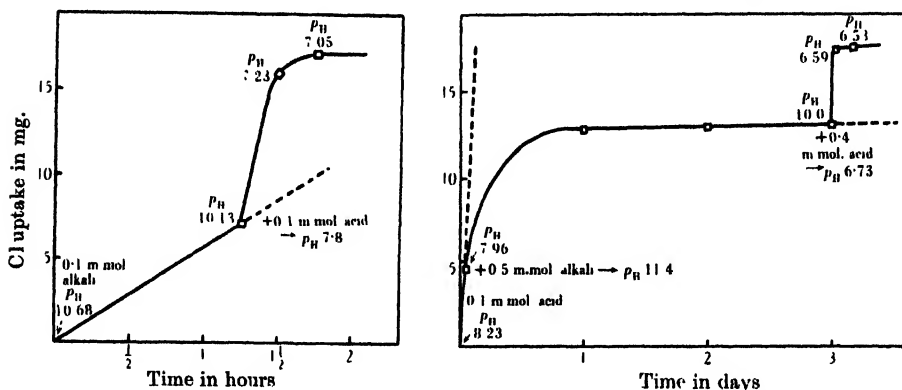


Fig. 8. Effect of adjustment of p_H on rate of oxidation (4 mg. glycine).

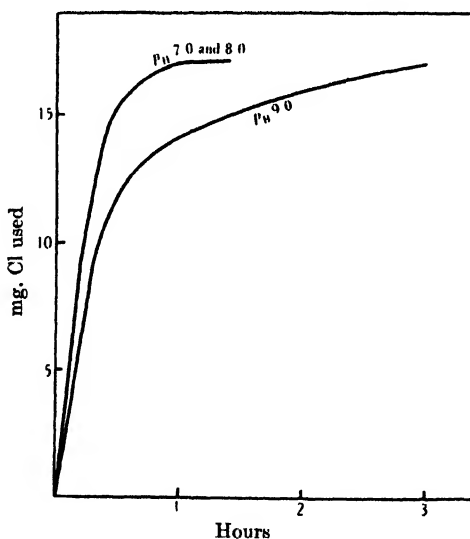


Fig. 9. Utilisation of Cl in buffered solutions.

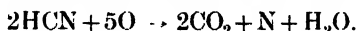
All observations show that, while the reaction may take place partially and slowly when the p_H is high, for complete oxidation approximate neutrality is required; retardation is also evident on the acid side. In order to determine the effect of stabilisation at a given p_H within the optimum range, solutions were buffered with Clark's [1928] phosphate and borate buffers to approximately p_H 7.0, 8.0 and 9.0, and titrated every 10 min. The solutions buffered at p_H 7.0 and 8.0 were very similar and are represented by the upper curve in Fig. 9. In the solution buffered at 9.0, while the initial rate of utilisation during the first half hour somewhat approximated to those in the other solutions, the rate subsequently decreased considerably so that 3 hours were required for complete oxidation of the glycine as compared with 1 hour for the other two.

The route of oxidation.

It has been experimentally determined above that 4.26 mg. of available Cl are utilised in completely oxidising 1 mg. of glycine (8.98 g.-atoms of available Cl per g.-mol. of glycine). Simple chlorination alone could account for but 4 atoms of available Cl. Dakin [1916] stated that the mono- and di-chloroamino-acids decompose spontaneously to give either the aldehyde and ammonia, or the nitrile respectively, CO_2 being liberated in both cases. In the case of glycine, HCHO or HCN would be formed by such decomposition, and using chloramine-T Dakin reported that he obtained traces of HCN from glycine. The uptake of 5 atoms of Cl by glycine remains unaccounted for, and it is evident that there must be a further utilisation of Cl by these initial fission products. Determinations of the amount of Cl used by ammonia, HCHO and HCN were therefore made.

(1) *Formaldehyde*. 2 mg. of formaldehyde were allowed to react with various amounts of sodium hypochlorite solution for 1 hour, 2 hours and 1 day, but in no case was there any loss of available Cl. This excludes the possibility of the formation of formaldehyde as an intermediate product.

(2) *Cyanide*. Solutions containing 1 and 2 mg. of KCN were allowed to react with various amounts of Cl as hypochlorite. The reaction was incomplete in 2 hours, but complete within 24 hours. The titration figures for nine samples varied from 2.8 to 3.15 mg. of Cl used per mg. of KCN present, with an average of 3.0 equiv. to 5.5 atoms of Cl per g.-mol. of cyanide. This is slightly in excess¹ of the theoretical figure of 5 atoms obtained from the following equation:



The reaction, however, is believed to be one initially of hydrolysis to formic acid and ammonia with the subsequent oxidation of these products.



(3) *Formic acid*. Preliminary experiments showed that H.COOH did utilise Cl at a slow rate. 5 mg. H.COOH and 11.2 mg. of available Cl (as sodium hypochlorite) per 100 ml. were allowed to react at room temperature. The amount of Cl used is given in Table VII.

Table VII. *Utilisation of chlorine by 5 mg. of formic acid.*

Time (days)	mg. Cl used
1	3.0
2	7.2
3	7.55
4	7.7
6	7.7

The uptake of 7.7 mg. Cl per 5 mg. of formic acid is equivalent to 2 atoms of Cl per mol. of acid. Evidently the formic acid is oxidised to carbon dioxide and water.

(4) *Ammonia*. 1 mg. of NH_3 was allowed to react with a large excess of Cl (30 mg.) for 2, 3 and 4 hours and 1 and 2 days. Oxidation is rapid and complete in 2 hours. The amount of Cl used is given in Table VIII.

The amount of Cl, 6.75 mg., used by 1 mg. of NH_3 is equivalent to 3.2 atoms of Cl for each mol. of ammonia, which agrees fairly well with the theoretical calculation for complete oxidation to nitrogen and water.

¹ The additional utilisation over and above the theoretical value is believed to be due to the formation of a small amount of nitrite, positive tests for which were obtained in certain cases. This point is being further examined.

Table VIII. *Utilisation of chlorine by 1 mg. of ammonia.*

Time	mg. Cl used
2 hours	6.85
3 "	6.75
3 "	6.75
4 "	6.75
1 day	6.6
2 days	6.65
2 "	6.85
Average	6.75

Thus of the possible intermediate products tested, cyanide, formic acid and ammonia have been shown to react with hypochlorite. The cyanide, most probably through the intermediate formation of formic acid and ammonia, accounts for the utilisation of 2 + 3 atoms of Cl. This, together with the 4 atoms of Cl required for the initial reaction with glycine, is in agreement with the uptake (by 1 g.-mol. of glycine) of 9 atoms of Cl as experimentally determined.

Carbon dioxide production.

During the reaction, there would be formed therefore, 2 mol. of CO_2 for each mol. of glycine oxidised, if the above theory of oxidation is true. Solutions containing 10 mg. of glycine, 25 ml. of phosphate buffer p_{H} 7.0 and 50 mg. of Cl, added as chlorine water, per 100 ml. of reaction mixture, were kept until the reaction was complete. Chlorine water was used instead of hypochlorite because the latter contained carbonate. After the reaction was complete the CO_2 was aerated off, absorbed in alkali and determined by back-titration. Three determinations gave 11.3, 11.6 and 11.5 mg. of CO_2 . 10 mg. of glycine would give a theoretical figure of 11.7 mg. of CO_2 .

The nature of the initial reaction.

There seems to be no clear evidence as to the nature of the initial reaction between an amino-acid and hypochlorite in excess. Two possibilities exist; either the amino-acid may be chlorinated to give, in the case of glycine, dichloroaminoacetic acid, or the acid may be directly oxidised to form the cyanide, with the liberation of CO_2 and water. Ammonia when treated with hypochlorite does yield chloroamine if the ammonia be present in excess [Chapin, 1929; 1931], an observation which indicates that even under such alkaline conditions hypochlorite may act as a chlorinating agent. Langheld [1909] and Wright [1926] stated that chlorination of the amino-group was the first stage in the action of hypochlorite on an amino-acid or protein in any proportion, and claimed that under certain conditions the dichloro-derivative of a monoamino-acid could be formed by simple mixture of the appropriate quantities of hypochlorite and acid. Since such chloroamino-derivatives are capable of liberating iodine from KI, no apparent loss of chlorine should be observed.

Repetition of Wright's experiment on this point, together with a similar experiment adjusted to neutrality, failed however to confirm his view (Table IX). A slow utilisation of chlorine was observed in the unaltered sample¹ and a more rapid one in the neutralised sample. Controls made at the same time showed a loss of only 0.2 mg. Cl in 3 days and 0.4 mg. in 10 days. These results may be more reasonably explained on the basis of a slow oxidation rather than as the breakdown of an unstable dichloro-compound, which would be expected to be more stable in the neutralised experiment than in the untreated experiment.

¹ Unaltered sample contained 0.17 m.mol. acid.

Table IX. *Utilisation of 14.3 mg. Cl by 15 mg. glycine.*

Time	mg. of available Cl used	
	Unaltered	Neutralised
2 hours	1.0	1.8
5 ..	1.2	3.5
17 ..	1.8	5.35
1 day	2.1	6.1
3 days	3.7	8.3
6 ..	6.15	10.45
10 ..	9.0	11.8

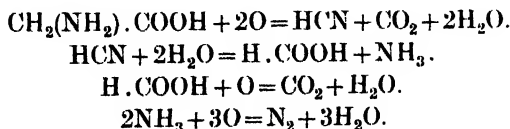
Some other evidence may be cited for the view that ordinary hypochlorite solution does not act as a chlorinating reagent. Cross *et al.* [1908] noted that gelatin treated with chlorine formed a chloro-derivative which may be washed, and subsequently titrated iodimetrically. However, gelatin immersed in hypochlorite retains very little titratable chlorine. Three amounts of 0.2 g. gelatin were allowed to stand with 60 mg. Cl as hypochlorite in 45 ml. water. To one experiment were added 5 ml. *N* acetic acid and to another 5 ml. *N* NaOH, the third receiving only 5 ml. water. After $\frac{1}{2}$ hour the liquid was drained off through fine cloth and the gelatin extensively washed till the washings gave no test for chlorine. This took several hours. The gelatin was then suspended in dilute acidified KI and the iodine liberated titrated in the usual way. The acid sample had retained 16.5 mg. Cl, the alkaline sample 1.4 mg. and the sample with hypochlorite alone 2.5 mg. Calculated on the nitrogen content of the gelatin (17 %) these results correspond to the chlorination of one NH group out of every five in the acid sample, one out of every 66 in the alkaline sample and one out of 37 in the unaltered sample. Chlorination is thus shown to be relatively small, except under acid conditions in which free chlorine would be present.

Indirect evidence against chlorination by hypochlorite is provided by the behaviour of lignified plant tissues after exposure to this reagent. No colour is given on subsequent treatment with cold sodium sulphite solution [Norman and Jenkins, 1933] whereas if acidified hypochlorite or gaseous chlorine be used a characteristic rich purple colour is obtained.

In the light of this rather conflicting evidence the nature of the initial reaction between an amino-acid and chlorine as hypochlorite in excess must remain open. The theory of direct oxidation to cyanide seems at least as well founded as that of the formation of chloroamino-acids.

CONCLUSION.

Whatever be the first step of the reaction, oxidation or chlorination, the reaction between glycine and an excess of hypochlorite probably results in the intermediate formation of hydrocyanic acid, which is then hydrolysed to formic acid and ammonia. These products may then be supposed to be completely oxidised with the liberation of carbon dioxide and gaseous nitrogen. The whole reaction may be given in the equations below, the possible validity of each of which has been experimentally verified.



SUMMARY.

1. Glycine is rapidly oxidised by hypochlorite. At least five times as much chlorine as glycine must be present for completion of the reaction. Under such conditions 1 mg. of glycine uses 4.26 mg. of chlorine, equivalent to 9 atoms of chlorine or $4\frac{1}{2}$ atoms of oxygen per mol. of glycine.

2. When there is a sufficient excess of chlorine to complete the reaction, the increase of chloride-chlorine is equal to one half of the amount of available chlorine used.

3. The rate of oxidation is most rapid and is complete in 2 hours between the acid and alkali concentrations of 0.05 m.mol. per 100 ml. (on either side). In general, further additions of acid or alkali greatly retard the rate of reaction. With alkali however a point of maximum retardation is reached at a concentration of 0.25 m.mol. per 100 ml. In the presence of alkali concentrations greater than this, the rate is very slowly increased, though the reaction is still not complete after a period of 25 days.

4. The change in p_H during the reaction has been followed by means of a glass electrode. The mixture becomes more acid as oxidation proceeds, and the reaction is most rapid in the region of p_H 7-9. Solutions buffered at p_H 7 and 8 are more rapidly oxidised than one buffered at p_H 9.

5. The oxidation of possible intermediate products in the reaction, $H.CHO$, HCN , $H.CO_2H$ and NH_3 , was tested. All except $H.CHO$ are completely oxidised by hypochlorite. From this it is established that the oxidation of glycine results first in formation of HCN , CO_2 and water. HCN is then hydrolysed to give formic acid and ammonia, both of which are oxidised to CO_2 , water and gaseous N . Quantitative recovery of carbon dioxide was obtained. In this way, the uptake of $4\frac{1}{2}$ atoms of oxygen per mol. of glycine is accounted for.

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LXXV. THE BIOLOGICAL OXIDATION OF CARBOHYDRATES.

V. THE DECOMPOSITION OF CELLULOSE IN THE ACTIVATED SLUDGE PROCESS AND IN PERCOLATING FILTERS.

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THE greater part of the dispersed matter in domestic sewage liquors has been shown by Mills [1931] to be present in the non-colloidal state. Cellulose is an important constituent of the suspended matter. Before sewage is purified by biological methods it is generally pre-treated by sedimentation to remove as much of the suspended solids as possible. Nevertheless a considerable proportion of the suspended substances is not separated by the pre-treatment and so passes on for purification by biological methods. The experiments described in this paper were designed to follow the course of decomposition of cellulose in two processes commonly employed for purifying sewage, *viz.* the activated sludge process and treatment in percolating filters.

EXPERIMENTS WITH ACTIVATED SLUDGE.

Tests were first made to see if an activated sludge, which would settle rapidly and leave a clear supernatant liquor, could be produced by aerating suspensions of cellulose in water to which ammonium carbonate had been added: these tests were not successful. A similar result followed the addition of suspensions of cellulose to an activated sludge derived from sewage and used successfully for the oxidation of solutions of sucrose. The method which proved to give the best, though by no means perfectly satisfactory, results was to add the suspensions of cellulose to an activated sludge built up from sewage solids. The defects in this method were that activated sludge made from sewage contains much extraneous matter and is difficult to sample accurately: the first defect was overcome by building up the activated sludge from the solids washed out of a percolating filter used for treating domestic sewage. The small amount of cellulose in these solids was ignored. The second defect was partly remedied, when the activated sludge had been built up by prolonged aeration of an aqueous suspension of the filter solids, by shaking the sludge vigorously in a measuring cylinder, allowing the heavy mineral matter to settle for a few seconds and then pouring off the creamy, homogeneous sludge.

The method of experiment was to aerate the sludge *plus* a known amount of suspension of cellulose, made up to a constant volume, for a definite period. The sludge was allowed to settle and the supernatant liquid and sludge were analysed. As the rate of disappearance of cellulose itself could not be followed with any

degree of accuracy in the concentrations of cellulose employed, determinations of organic carbon were made and the carbon figures converted into amounts of cellulose by using a factor.

Analyses made. Determinations of organic carbon, total nitrogen, nitrogen as ammonia, nitrite and nitrate, and total solids were made on the supernatant liquors. Organic carbon was determined by Mills's method [1931] in the early stages of the experiment, absorbing the CO_2 in baryta solution contained in a Pettenkoffer tube, and back-titrating the baryta with standard acid. For the purposes of this experiment, however, the method of Robinson *et al.* [1929] was later found to be more suitable. The liquids were evaporated under reduced pressure to a small bulk and then digested with H_2SO_4 in a 300 ml. Kjeldahl flask fitted with a well ground-in head carrying two fused-in tubes. One tube projected about half-way into the bulbous part of the flask and was connected to a Saja air pump. The other tube, which did not project into the neck of the Kjeldahl flask, was connected to a Reiset tower fitted in a 250 ml. filter-flask. In the determination, organic carbon reduces the sulphuric acid and liberates sulphur dioxide, which is absorbed in standard iodine solution. In order to catch iodine volatilised during the passage of the gases through the iodine in the filter flask, the latter was connected to a trap containing a known volume of standard thiosulphate. Using a preparation of pure cellulose a quantity which in theory contained 10.63 mg. C was found to contain 10.29 and 10.97 mg. in duplicate determinations.

Total nitrogen was determined by the Kjeldahl method. When appreciable amounts of nitrite or nitrate were present, they were reduced with Devarda alloy and then distilled with magnesia into standard acid; organic nitrogen was determined in the residue. Ammoniacal nitrogen was determined by distillation with magnesia, nitrite colorimetrically by the *m*-phenylenediamine method.

In order to study the influence of an available form of nitrogen on the rate of disappearance of cellulose, two experiments A and B were made. In A, cellulose and nitrogen were always added to the sludge in the proportion of approximately 8 of C to 1 of N. In B the same amount of cellulose was added, but only one-tenth of the amount of N. These C/N ratios were used so that comparisons could be made with previous tests [Jenkins, 1933, 2] using sugar. The C/N ratios used over the whole experimental period of 153 days were 8.35/1 for Exp. A and 82.6/1 for Exp. B.

At intervals varying from 3 to 12 days, aeration was stopped and the liquid was allowed to settle for 1 hour. Supernatant liquid equal to 80 % of the total volume was then removed and replaced by an equal volume of water containing cellulose and nitrogen in the right proportion; aeration was then continued.

Solids washed out of a percolating filter, as previously mentioned, were used at the start of the experiments. These solids were aerated with a mixture of crude sewage and cellulose until a sludge which settled rapidly and left a clear supernatant liquid was produced. This sludge was used in the proportion of 200 ml. sludge to 800 ml. cellulose suspension. The cellulose used throughout these experiments was a special preparation kindly supplied by the Northfleet Paper Mills, Ltd., Gravesend, and had been made by carefully drying paper pulp wetted with dilute HCl: the dried pulp after extraction with dilute NaOH was washed free from salts. This preparation contained no fibre and remained as a paste for nearly a year without undergoing any change. The dried cellulose contained 44.4 % of carbon.

The compositions of the shaken liquids at the beginning of the experiment and after aerating for 7 days are given in Table I.

Table I. *Results of carbon and nitrogen determinations before and after aeration for 7 days.*

		(g. per litre.)					
Approximate C/N ratio	Time	Organic carbon			Total nitrogen		
		In sludge	In cellulose added	In sewage added	In sludge	In sewage added	In NH_3 added
8/1 Exp. A	At start	2.27	0.45	0.04	0.331	0.028	0.047
8/1 Exp. A	After 7 days		Total 2.76			Total 0.406	
80/1 Exp. B	At start	2.27	0.45	0.04	0.331	0.028	0.005
80/1 Exp. B	After 7 days		Total 2.76			Total 0.364	
Distribution of N.							
		Total N	$\text{NH}_3\text{-N}$	$\text{NO}_3\text{-N}$	$\text{NO}_2\text{-N}$	Organic-N	
8/1 Exp. A	After 7 days	0.381	0.036	0.030	0.0004	0.309	
80/1 Exp. B	After 7 days	0.343	0.039	0	0	0.304	

A gain of carbon was found in Exp. A and a loss in Exp. B, but as both gain and loss were within the experimental error of the determination, it is assumed that the carbon content remained the same before and after aeration for the first 7 days. The liquids were allowed to settle after this period of aeration and a known volume of supernatant liquor was removed and analysed. This liquid was replaced in the manner previously described. An attempt was thus made to keep a complete carbon and nitrogen balance sheet. The numerous data are not shown in detail but the losses of carbon are given in Table II for each period of aeration. A negative sign means that an increase in the carbon content was found. Analytical and sampling errors are in all probability responsible for the apparent gains in carbon content. The net loss of carbon for the total period of 153 days is the sum of the positive and negative losses.

Table II.

Days aerated 0 = beginning of exp.	Exp. A g. carbon lost	Exp. B g. carbon lost
0 - 7 = 7 days	0.11	+ 0.05
7 - 19 = 12 ..	+ 0.51	+ 0.11
19 - 24 = 5 ..	0.10	0.00
24 - 27 = 3 ..	+ 0.25	+ 0.32
27 - 35 = 8 ..	- 0.21	- 0.21
35 - 38 = 3 ..	+ 0.01	0.00
38 - 89 = 51*	+ 0.08	+ 0.07
89 - 97 = 8 ..	0.00	0.00
97 - 109 = 12 ..	+ 1.24	+ 1.69
109 - 117 = 8 ..	+ 0.20	+ 0.20
117 - 126 = 9 ..	0.00	+ 0.18
126 - 133 = 7 ..	+ 0.71	+ 0.30
133 - 140 = 7 ..	+ 0.18	+ 0.05
140 - 145 = 5 ..	+ 0.11	+ 0.17
145 - 153 = 8 ..	0.08	+ 0.20
Net loss	+ 2.79	+ 3.13

* An accident prevented analyses and additions of cellulose *etc.* being made from the 38th to the 89th day. The development of green algal growths during this period probably accounts for the small loss of carbon recorded.

Over the whole period the vessel which received additions of C and N in the ratio of 80/1 showed a somewhat greater loss of C than the one receiving more N. In all 5.17 g. of C as cellulose were added in each experiment, of which 54% was removed in Exp. A and 60% in Exp. B. But in view of the unaccountable

variation in the amount of C oxidised during similar periods of time, it cannot be stated with certainty that the differences between the total amounts of C oxidised in Exps. A and B are really significant. The conclusions can safely be drawn, however, that (1) cellulose in a fine state of division is removed from suspensions aerated for several days with a sludge derived from sewage: (2) the cellulose removed from suspension is oxidised, at least in part, during aeration: (3) a very small ration of nitrogen is required for the oxidation of cellulose by the activated sludge process. No advantage resulted from adding more N than was provided when the C/N ratios of the liquids added were about 80/1. It may be that the effect of adding little nitrogen in proportion to cellulose was to stimulate the activity of nitrogen-fixing organisms, as is known to occur in soil [Beijerinck, 1904], and in percolating filters fed with solutions of sugar and small amounts of nitrogen [Jenkins, 1933. 2]. However, the well-known effect of available nitrogen in stimulating the decomposition of cellulosic substances in the soil, or the activity of cellulose-decomposing organisms such as *Spirochaeta cytophaga* [Hutchinson and Clayton, 1919], does not appear to apply to the same extent in the case of cellulose breakdown in the activated sludge process.

The liquid in Exp. A generally contained a considerable proportion of nitrogen as ammonia at the end of the periods of aeration. When the concentration of ammonia at this stage was low, a correspondingly large amount of nitrogen was found as nitrate and sometimes as nitrite. In Exp. B ammonia was frequently present in small amounts. At times, however, the whole of the nitrogen added as ammonia was found after aeration to be in the organic form. Only on one occasion was a trace of nitrite detected and on another a trace of nitrate. As cellulose and nitrate co-existed in Exp. A without the nitrate disappearing it is clear that the assimilation of nitrate during the microbiological decomposition of cellulose takes place much more slowly in the activated sludge process than in the soil [Murray, 1921]. To illustrate the difference in concentrations of various compounds of nitrogen in Exps. A and B typical results of analysis are given in Table III.

Table III. *Results showing the difference in concentration of nitrogenous compounds in Exps. A and B.*

Period of experiment	(Parts per 100,000.)					
	Exp. A			Exp. B		
	Ammonia	Nitrite	Nitrate	Ammonia	Nitrite	Nitrate
19th day	4.67	0.03	2.15	1.76	0	0
After aeration from 19th to 24th day	0.03	0	3.20	0	0	0
27th day	2.93	0.02	0.77	0.31	0	0
After aeration from 27th to 35th day	0.12	0	1.43	0.09	0	0
89th day	4.23	--	0.14	0.34		0.01
After aeration from 89th to 97th day	0.47	0	0	0.14	0	0
117th day	4.91	0	0.09	0.50	0	0
After aeration from 117th to 126th day	2.05	0	2.50	0.15	0.06	0
133rd day	5.06	0	0.36	0.49	0	0
After aeration from 133rd to 140th day	1.73	0	2.31	0.03	0	0

for the deviations in the concentration of carbon in the nutrient liquids given in Table V, from the amounts shown in Table IV.

A few determinations of carbon and nitrogen in various forms were made at different levels in the filters. The results are shown in Table VI.

Table VI. *Organic carbon and ammonia in liquids fed to Filters C and D and in the effluents from different sections.*

Filter C, C:N ratio 8.8/1; Filter D, C:N ratio 88/1. Parts per 100,000.

Day of exp.	Nutrient liquid	Filter C				Nutrient liquid	Filter D			
		Section					Section			
		1	2	3	4		1	2	3	4
Organic carbon.										
35	29.1	16.0	6.9	6.4	16.1	24.2	14.5	7.2	9.9	-
117	27.4	6.7	-	5.2	4.9	25.5	5.7	-	3.6	3.9
131	39.8	3.8	-	3.8	4.3	27.3	3.6	-	3.8	5.1
141	41.1	6.3	-	17.0	12.1	39.6	20.1	-	1.8	1.4
153	63.2	12.2	10.9	8.7	5.7	41.1	9.3	4.3	2.9	4.9
N as Ammonia.										
35	2.25	2.0	1.5	1.25	1.30	0.2	0	0	0	0
117	2.3	2.0	-	1.75	1.65	0.24	0	-	0	0
131	2.3	1.75	-	1.75	1.75	0.24	0	-	0	0
141	4.0	-	4.0	3.0	3.0	0.4	Trace	-	0	0
153	5.74	5.04	4.91	4.16	4.58	0.53	0.03	0.03	0.03	-

At the end of the filtration experiments the composition and amount of the solids in all four sections of Filters C and D were determined. The solids were removed by shaking the tubular glass medium in the filters with hot water. The results are summarised in Table VII in which the appearance of the film is also noted.

Table VII. *Filter C, C/N ratio 8.8/1; Filter D, C/N ratio 88/1.*

Weight of solids, C, N and NH₃, found in sections at end of experiment (g.).

	Filter C					Filter D				
	Section				In all sections	Section				In all sections
	1	2	3	4		1	2	3	4	
Total solids	3.03	1.83	1.08	0.47	6.41	13.00	7.60	3.41	1.93	25.94
Combustible solids	3.03	1.82	1.07	0.47	6.39	12.81	7.34	3.13	1.91	25.19
Ash	0	0.01	0.01	0	0.02	0.19	0.26	0.28	0.02	0.75
Organic C	1.34	0.78	0.45	0.24	2.81	5.38	3.33	1.25	0.75	10.71
Total N	0.050	0.044	0.031	0.023	0.148	0.081	0.071	0.061	0.057	0.270
NH ₃ -N	0.006	0.005	0.001	0.001	-	0.005	0.002	0.001	0	-
Organic N	0.044	0.049	0.030	0.022	0.145	0.076	0.069	0.060	0.057	0.262
Organic C Org. N	30.1	16.1	15.1	11.1	-	71.1	48.1	21.1	13.1	-
	Section					Section				
	1	2	3	4		1	2	3	4	
Colour ivory-brown. Texture like original cellulose pulp; slimy. <i>Colembola</i> , worms, <i>Psychoda</i> flies and larvae present		Colour as 1. Less film than in 1. Worms and <i>Colembola</i> abundant	As 2, but less in amount	Little film, mostly worms and <i>Colembola</i>		Ivory colour. Thick algal growth on side of filter. Protozoa abundant	As 1, but less in amount	Ivory colour. Like cellulose in texture		Much algal growth

Discussion of filtration experiments.

With two exceptions the figures giving the amount of carbon in the effluents (Table V) show little difference between the two filters. It would be wrong to conclude from this fact that the filter receiving C and N in the ratio of 88/1 was as effective in decomposing cellulose as the other filter, receiving C and N in the ratio of 8.8/1. Whilst the amounts of carbon in the effluents were of the same order, the quantities which accumulated in the filters were quite different. The first section of Filter C was the only one which contained much growth or deposit. The first three sections of Filter D, however, were thick with an ivory-white deposit. The first section of this filter contained enough film to impede filtration in the later stages of the experiment. The amounts of total solids and organic carbon in the various sections agreed with the appearances of those sections. The figures show a descending order of solid matter and organic carbon in passing from section 1 to section 4. They also show that each section of Filter D had about four times as much solid matter as the corresponding section in Filter C. There was 6.5 % of ash in the solids from Filter C and only 2.8 % in those of Filter D. It is worth recording that part of the film often passed in flushes from the bottom of one section to the top of the section immediately below. This appeared liable to occur at any stage of the experiment, as the film lacked cohesion. The amounts of carbon in the effluents from different sections (Table VI) show that the greater part of the carbon removed was deposited in the first sections of the filters. Inspection indicated that the film in the lower sections in both filters originated from pieces broken away from the film in the upper sections and washed down.

The results of determinations of nitrogen in its various forms are of interest. Although Filter C received 10 times as much N as ammonium bicarbonate as Filter D, the amounts of organic nitrogen in the films at the end of the experiment were: 0.145 g. in C, and 0.262 g. in D. Assuming that the microbial C and N were present in the ratio of 10/1, the amounts of C in the two filters in this state would be 1.451 g. and 2.622 g. in Filters C and D respectively, leaving 1.358 and 8.079 g. C in the non-biological form in C and D respectively. A large proportion of this non-microbial C was probably undecomposed or only partly decomposed cellulose.

Filter D removed ammonia quantitatively from the nutrient liquid, except on the 153rd day. The amount of ammonia taken up by Filter C varied between twice and four times as much as that retained by D, yet, as already pointed out, the film in Filter D contained more N than Filter C. Most of the N taken from the nutrient liquid was removed by the first section, as was the case with carbon. The first section of Filter D extracted quantitatively all the N supplied, with the one exception mentioned. Nevertheless, a relatively large amount of N appeared in the remaining sections of this filter derived from the film in Section 1.

The information required to draw up a balance sheet between C and N supplied and the amounts of these elements found in the effluents and the filter is incomplete. By assuming that the analytical results obtained whenever samples were taken are typical of the behaviour of the filters between different times of sampling, the following approximate balance sheet may be drawn up.

	Filter C. C/N ratio 8.8/1		Filter D. C/N ratio 88/1	
	C	N	C	N
Added	56.3	6.62	56.3	0.66
Found in effluents	14.1	4.78	8.9	0.03
Found in filter	2.8	0.15	10.7	0.27
Unaccounted for	39.4	1.69	36.7	0.36

The accuracy of the figures for C and N unaccounted for depends upon the assumption, made in the previous paragraph, that the composition of the final effluents remained constant over fairly long periods. The variation in composition of the effluents suggests that normal fluctuations would not greatly affect the estimates of C and N lost. The C lost during filtration probably disappeared as CO_2 , since it did not appear in the effluents as soluble organic carbon. Although the amounts of carbon oxidised by Filters C and D may not be significantly different, the differences in the carbon content of the filters are undoubtedly associated with the differences in the nitrogen supplied to the filters. Most of the carbon in Filter D was probably undecomposed cellulose.

The nitrogen used in the filtration of suspensions of cellulose depends upon the amount of nitrogen supplied. In presence of a large excess of nitrogen, 39.4 g. C utilised 1.7 g. N; with a small supply of nitrogen 36.7 g. C used only 0.36 g. N. Under the conditions of these experiments the nitrogen requirement of a filter is not an absolute figure but is capable of variation within wide limits. With different conditions Hutchinson and Richards [1921] found in the case of decomposition of straw that favourable decomposition occurred with an initial C/N ratio of about 40/1.

SUMMARY.

Aqueous suspensions of cellulose, containing different amounts of N as NH_4HCO_3 , were treated by the activated sludge process in one set of experiments, and in glass percolating filters in another.

Activated sludge experiments. Approximately the same amount of cellulose was oxidised when cellulose and nitrogen were added in the C/N ratio of about 80/1 as when the C/N ratio was 8/1. The cellulose did not disappear at a steady rate, for on occasions more was lost during short periods than during longer periods of aeration.

The oxidation of NH_3 to nitrite and nitrate was inexplicably erratic when the C/N ratio was approximately 8/1 and bore no apparent relationship either to the amount of cellulose oxidised or to the period of aeration. Cellulose and ammonia were oxidised simultaneously. There was no evidence to show that the decomposition of carbohydrate preceded the oxidation of the nitrogen.

Filtration experiments. When cellulose and nitrogen were supplied to two filters in the ratio of about 8/1 and 80/1 respectively it appeared that about 10% more decomposition occurred with the lower ratio; the difference may not be significant. The two filters differed in appearance at the end of the experiment. The one receiving cellulose and much N had enough active film to decompose 70% of the C it received and allow liquid to percolate freely; the other, receiving the same amount of cellulose and less N, oxidised 65% of the C, contained nearly four times as much film as the first filter and did not allow the free passage of liquid.

Of the N supplied to the filter receiving C and N in the ratio of 8/1, 72% of the N was recovered as NH_3 in the effluent, 2% in the filter and 26% disappeared. The absence of N from the effluents from the filter receiving C and N in the ratio of about 80/1 showed that practically all was probably retained by the filter. However, out of the small amount of N supplied, only 45% was accounted for in the film and in the effluents.

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LXXVI. GALACTOZYMASE CONSIDERED AS AN ADAPTIVE ENZYME.

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PREVIOUS studies from this department on adaptive enzymes have been concerned with hydrogenlyases. It has been shown that the formation of these enzymes in the Bacteriaceae is controlled by the medium in which the cell is grown, one factor being the presence of the substrate, *e.g.* formate in the case of formic hydrogenlyase, another being some substance in broth which so stabilises the enzyme that it is active in washed suspensions [Stephenson and Stickland, 1932; 1933; Yudkin, 1932]. The mechanism by which the medium conditions the formation or stabilisation of enzymes was discussed by Yudkin [1932], who postulated two possibilities, either natural selection or chemical adaptation, *i.e.* direct chemical action of the substrate on the cell. He concluded, chiefly from theoretical considerations, that the production of formic hydrogenlyase by *Bact. coli* was of the nature of a chemical adaptation. This was later proved by Stephenson and Stickland [1933], who showed that natural selection was not operating, since the enzyme was formed in the absence of cell multiplication. In the present study we present some facts connected with the formation of galactozymase, the enzyme complex concerned with the fermentation of galactose by *S. cerevisiae*.

The literature already contains material on this subject. Dienert [1900] first recorded the adaptive nature of galactozymase by showing that certain strains of yeast, which at first are unable to grow in media where galactose is the sole carbohydrate, become able to do so if previously grown for a few weeks on a mixture of glucose and galactose. In such cases the same result is achieved by lactose or melibiose, doubtless because these sugars give galactose on hydrolysis. Yeast grown on glucose alone is unable to ferment added galactose, whilst yeast grown on galactose or on a mixture of glucose and galactose, when subsequently placed in contact with galactose, causes an immediate fermentation of that sugar, *i.e.* is adapted to galactose. Non-adapted yeast if left in contact with galactose for 24 hours or longer begins a slow fermentation; after this is complete, a further addition of galactose is fermented immediately, *i.e.* the yeast has become adapted. Moreover, adapted yeast, if allowed to ferment glucose, showed subsequently a diminished power to ferment galactose, that is, adaptation is partly reversible. This observation was confirmed by Euler and Johansson [1912] and Euler and Lövgren [1925] and by Söhngen and Coolhaas [1924], and contradicted by Abderhalden [1926, 2].

These experiments of Dienert, though highly suggestive, are difficult to interpret owing principally to their non-quantitative nature, rates of fermentation, quantity of yeast and change in cell numbers not being recorded.

Slator [1908] showed that only certain strains of yeast can be adapted to galactose. He was of opinion that the fermentation of galactose does not occur through glucose or through a form common to both sugars, since unadapted yeast when added to adapted yeast does not increase the rate of fermentation of galactose.

Harden and Norris [1910] found galactozymase in the press juice from adapted but not from unadapted yeast, thus disposing of the idea that adaptation consists in increasing the permeability of the cell to galactose. They also showed that the rôle of phosphate in galactose fermentation is similar to that in glucose fermentation.

Abderhalden [1926, 2] showed that dried adapted yeast retains its fermentative power for long periods; he claimed also to have adapted dried yeast, but this was probably due to the adaptation of surviving cells which multiplied during the process. He also showed [1926, 1] that adaptation is not due to the formation in the medium of substances accelerating the fermentation of galactose, whilst Euler and Jansson [1927] excluded the possibility that adaptation is concerned with the formation or modification of cozymase.

Evidence on the connection between adaptation and viability is conflicting. Söhngen and Coolhaas [1924] carried out adaptation at 30° and measured fermentation at 38° in order to avoid cell multiplication during fermentation. They found that the rate of fermentation was proportional to the number of new cells formed and concluded that the new cells formed during adaptation are alone responsible for the fermentation. They found that, when adapted yeast ferments glucose, the adaptation is partially reversed, but that this process is independent of multiplication. No adaptation occurred in two weeks at 0°. Euler and Johansson [1912] stated that adaptation occurs only in the presence of galactose and asparagine and certain salts; later Euler and Nilsson [1925] found that, though adaptation does not occur in galactose and water, it does so if the "Z" factor is added. This evidence is on the whole in favour of adaptation being associated with growth. Abderhalden on the other hand [1926, 1] supported Dienert in showing that adaptation occurs in an aqueous solution of galactose and stated on microscopic evidence that growth did not occur.

The evidence obtained by adapting yeast in the presence of growth inhibitors is untrustworthy. Euler and Nilsson [1925] tried to adapt yeast in the presence of about 0.5% phenol, and found that after the removal of the phenol the yeast fermented galactose after a latent period of about 15 hours. In the absence of sterility tests or of viable cell counts this evidence is inconclusive. Similar experiments were done with alcohol-treated dried yeast. This work was criticised by Söhngen and Coolhaas [1926], who showed that, in the conditions of Euler and Nilsson's experiments, cell multiplication occurs when the yeast poisoned by phenol or by alcohol is subsequently placed in contact with galactose. Euler and Jansson [1927] accepted the criticisms of Söhngen and Coolhaas, and modified their original experiment by carrying out the fermentation in the presence of phenol, with negative results. They also attempted to dissociate growth and adaptation by carrying out the latter process at 38°; at this temperature neither growth nor adaptation occurred.

The following work was done to determine the exact conditions in which galactozymase is formed and the relation between the formation and activity of the enzyme complex with the multiplication and viability of the cell.

TECHNIQUE.

The yeast used was a pure strain of *S. cerevisiae* obtained from the National Collection of Type Cultures. It was grown in Roux bottles on a tryptic digest of caseinogen of half the strength used for bacteria with 1% of glucose (or galactose) and at a p_H of about 6.0. The bottles were incubated at 28°; in the earlier experiments the growth period was 2–3 days, but later this was extended to 6 days for reasons given on p. 509. The yeast was centrifuged and washed twice with sterile

water; the crop from 1 l. was made up to about 15 ml. The fermentative power of the suspension was measured by the rate of carbon dioxide evolution in a Barcroft manometer. In the right-hand cup were placed 1 ml. of phosphate buffer p_{H} 6.0, 1 ml. of 5 % sugar solution and 1 ml. of yeast suspension; in the left-hand cup the sugar solution was replaced by water. The apparatus was evacuated twice and filled with nitrogen previously passed over heated copper. The fermentations were done at 30°. Sterile precautions were observed throughout except in the manometers.

All samples of galactose (including Kahlbaum's) tested by us contained enough glucose or other fermentable sugar to give a measurable fermentation with untrained yeast in the Barcroft apparatus. We purified our galactose by a modification of the method by which Somogyi [1927] removed glucose from blood by adsorption by yeast. 100 g. of baker's yeast were washed in 1 l. of distilled water and centrifuged; this process was performed 4 times. The washed yeast was then suspended in 100 ml. of 20 % galactose, made up to 200 ml., left for 10 min. and centrifuged; 100 ml. of the supernatant fluid were then treated with 50 g. of washed yeast as before and the process exactly repeated. 100 ml. of the final supernatant fluid were treated with 8 ml. of "colloidal iron" and filtered twice through kieselguhr. The final solution contained between 4 and 5 % of galactose, and 1 ml. of this solution was used in the Barcroft cup for each fermentation experiment.

The total counts were made after suitable dilution by means of a Zeiss Thoma slide as used for blood counts. The viable counts were made by the roll tube method as described by Wilson for bacteria [1922]. The broth used for the agar medium was identical with that used for growing the yeast, with about 0.05 % of marmite (wet weight) added in order to increase the size of the colonies. Incubation was at 28° and the counts were made at the end of 3 or 4 days.

Preliminary experiments.

In accordance with previous observers we found that our yeast when grown on glucose broth failed to ferment galactose; when grown on galactose broth it fermented both sugars. For example, a suspension of yeast grown on glucose broth fermented galactose at a rate of 280 $\mu\text{l.}/\text{hr.}$ and failed to ferment galactose at a measurable rate. Another sample grown on galactose broth fermented glucose at 830 $\mu\text{l.}/\text{hr.}$ and galactose at 490 $\mu\text{l.}/\text{hr.}$ The ratio of the rates of galactose to glucose fermentation was thus about 0.6 and this ratio was generally obtained. Previous observers have obtained ratios from 1.2 [Harden and Norris, 1910] to 1.6 [Dienert, 1900; Willstätter and Sobotka, 1922], but in these cases the conditions of growth differed from ours.

Conditions affecting the formation of galactozymase.

The yeast from 900 ml. of glucose medium was grown, centrifuged and washed in the usual way, and the suspension made up to 15 ml. Into 4 graduated centrifuge-tubes was put 1 ml. of yeast suspension together with galactose and other reagents (see Table I).

Table I.

Yeast suspension ml.	5% galactose ml.	Inorganic medium ml.	1% asparagine ml.	Ringer's solution ml.	Phosphate buffer p_{H} 6.0 ml.	Water ml.	Fermentation rate $\mu\text{l. CO}_2/\text{hr.}$
1	1	1	0	0	0	2	104
1	1	1	1	0	0	1	100
1	1	0	0	1	0	1	117
1	1	0	0	0	1	1	115

The inorganic medium contained ammonia as source of nitrogen [Stephenson, 1930, p. 275, vi]. Each centrifuge-tube was incubated for 2 days, centrifuged and the yeast washed and made up to 3 ml.; the fermentation rate of the yeast was then determined. It appears from these results that the nitrogenous constituents play no part in the adaptation process, the presence of galactose alone being the determining factor. The effect of temperature on adaptation is shown in Table II.

Table II.

Temperature during adaptation ° C.	Rate of fermentation of galactose $\mu\text{l. CO}_2/\text{hr.}$
0	0
20	18
30	63
37	60

Adaptation occurs independently of oxygen. A yeast suspension was treated with galactose for 2 days; in the first case the reaction was carried out in a centrifuge-tube as usual; in the second case air was bubbled continuously through the centrifuge-tube; in the third case the conditions were anaerobic. The subsequent rates of galactose fermentation ($\mu\text{l. CO}_2/\text{hr.}$) were 230, 250 and 200 respectively.

Association of enzyme production and growth.

From the previous experiment it is seen that in the presence of galactose the washed yeast suspension acquired the enzyme as well in water or Ringer's solution as in the presence of a source of nitrogen. This raises the question whether yeast must grow in order to produce the enzyme. We attacked this problem by several methods; in the first place by making a series of measurements of enzyme activity associated with total cell counts. In each of 6 graduated centrifuge-tubes were placed with sterile precautions 3 ml. of 5 % galactose solution, 3 ml. phosphate buffer at p_{H} 6.0 and 2 ml. of yeast suspension; the tubes were incubated at 28°. At intervals of 24 hours one tube was withdrawn, centrifuged and the yeast washed and made up to 3 ml.; 0.5 ml. was withdrawn for counting and the remainder used for the determination of fermentation rates. From Fig. 1 it is seen that the total number of cells remains approximately constant whilst the enzyme activity rises to a peak on the second day and then falls off. In a similar experiment (Fig. 2) the galactozymase rises to a peak on the first day while the cell count remains constant, after which the activity falls off while the cell count rises. From these two experiments it appears that enzyme formation is not necessarily accompanied by cell multiplication. These results are however not perfectly conclusive. In the first place, the proportion of viable to total cells in different experiments may vary from 5 to 50 %. Where the ratio is low it is possible that the viable cells may increase 100 % whilst causing an increase of only 5 % on the total, the increase thus falling within the experimental error; for this reason, in most of our experiments we employed a viable count. During many of the experiments described later we found a definite increase in viable cells during adaptation, whilst in others they remained constant and sometimes decreased. Finally we chanced on the explanation for these contradictory results. In our earlier experiments the yeast was grown for 2 or 3 days, by which time the glucose in the medium was not completely fermented, and the yeast therefore in a good state of nutrition. If the growth period is prolonged to 6 days the glucose is exhausted and the yeast deprived of much of its store of nutrients; in these circumstances adaptation can

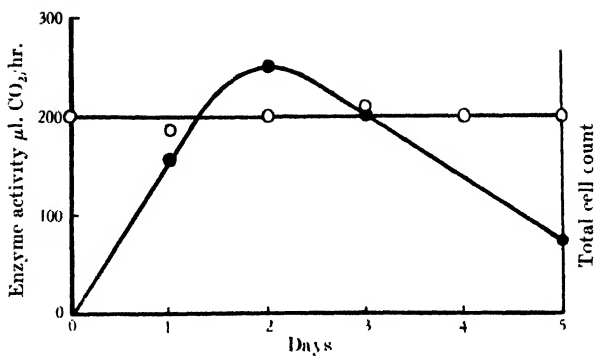


Fig. 1. ●—● Enzyme activity; ○—○ total cell count.

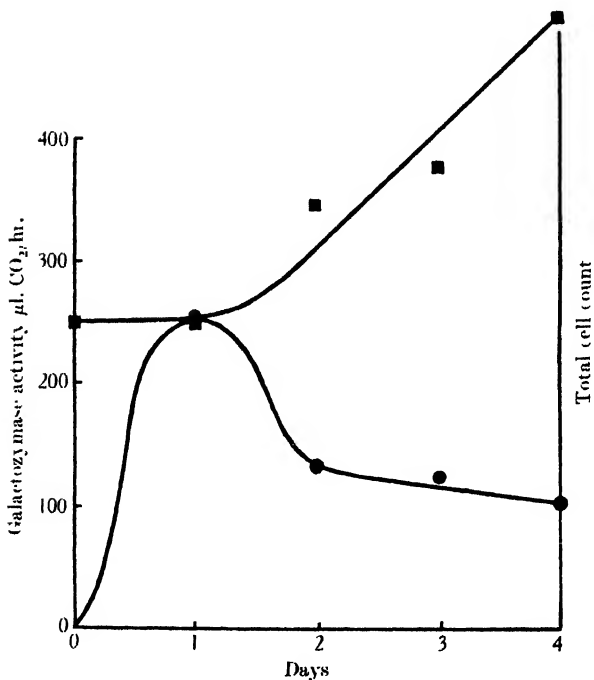


Fig. 2. ●—● Enzyme activity; ■—■ total cell count.

Table III. *Relation between adaptation and growth.*

Exp.	Before adaptation	After adaptation	Increase %
	Viable cells/ml. $\times 10^7$.		
1	4.5	2.7	-40
2	3.7	4.0	+ 8.1
3	5.17	5.4	+ 3.8
	Total cells/ml. $\times 10^7$.		
4	17.8	17.9	+ 0.6

be obtained repeatedly without any increase in viable count (see Table III). In order to meet the objection that the cell multiplication is actually occurring but balanced by an equal number of deaths a total count was also included.

These experiments prove that natural selection plays no part in the formation of galactozymase, which furnishes another example of chemical adaptation.

Association of enzyme formation and viability.

The next point we tried to determine was whether enzyme formation can occur in "dead," by which we mean non-viable, cells. We first attacked this problem by poisoning a culture to the point of complete sterility with various poisons, and then subjecting it to the adaptive process in the usual way. In these experiments we obtained negative results throughout. The following poisons were tried: saturated solutions of toluene and of chloroform, 2% propyl alcohol, mercuric chloride *M*:500 to *M*:2000 and 2% cyclohexanol. It was found that, where enzyme formation occurred, subcultures of the suspension into glucose broth invariably grew, and where complete sterility occurred, no enzyme formation took place.¹

A statistical study of viability and galactozymase activity was next undertaken by the use of graded doses of ultraviolet light. The first step was to determine the relation between the activity of galactozymase once formed and the number of viable cells. Yeast from 750 ml. of medium was centrifuged and washed, made up to 14 ml. and transferred to a sterile bottle of 150 ml. capacity containing about 50 glass balls about the size of a pea; the bottle was closed with a glass stopper, sealed with hot paraffin wax and shaken on a mechanical shaker for 2 hours. If (as in Exp. 2, Table V) the shaking is prolonged to 12 hours, the proportion of viable to total cells becomes decreased. After shaking, 6 ml. of yeast suspension + 1.5 ml. of phosphate buffer pH 6.0 + 1.5 ml. of 5% galactose solution were placed in each of two graduated centrifuge-tubes and incubated at 28° for 2 days. The tubes were then centrifuged and the yeast washed once and made up to 6 ml.; 1 ml. was removed and diluted for counting and 3 ml. for fermentation, and the remainder exposed in a double quartz tube with a water jacket 6 in. from a mercury lamp; details of this procedure are given in a previous paper [Cook and Stephenson, 1928]. Two exposures each of 5 min. were given; after each exposure 1 ml. was withdrawn for a viable count and 3 ml. for fermentation (Table IV).

Table IV. *Effect of irradiation by ultraviolet light on viability and on galactozymase.*

Time of exposure, min.	0	5	10
Total count, cells/ml.	17×10^7	17×10^7	17×10^7
Viable count, cells/ml.	(a) 3.6×10^7	2.3×10^5	0
Rate of fermentation of galactose, μ l. CO ₂ hr.	(R) 259	134	60
	(R/a) 1.98×10^{-1}	5.83×10^{-2}	/

From this it is seen that with an initial ratio of viable to total cells of 0.47 the fermentation rate is 259; when 1 in 10,000 only of these cells is viable the fermentation rate is reduced to about one-half, and with complete sterility the rate is still one-fifth of the original. Hence it is clear that the action of

¹ A similar result has been recorded with the formation of the adaptive bacterial enzyme decomposing the specific polysaccharide of the Type III Pneumococcus [Dubos, 1935]. Here the question of cell multiplication is undecided, but no enzyme formation occurs in the presence of antiseptics.

galactozymase, once formed, is independent of viability. Experiments with glucozymase gave similar results.

The relation between the formation of galactozymase and viability was next attacked. Two possibilities occurred to us. The formation of the enzyme may be conditioned by the number of viable cells present before adaptation, or by the number present after adaptation, supposing—as in our earlier experiments—some increase or decrease to have occurred.

In order to test these possibilities yeast suspension was made in the usual way and total and viable counts were made. One portion was then exposed to ultraviolet light for 5 min., and another for 10 min. A sample from each was then withdrawn for a viable count and the remainder incubated with buffer and galactose for 2 days. A second viable count was then made and a fermentation rate with galactose determined. The results of two experiments are given in Table V.

Table V.

Cells/ml. $\times 10^6$	Exp. 1	Time of exposure (min.)			
		0	5	10	
Total before adaptation		190	—		
Viable before adaptation	(a)	91	9.1	0.59	
Viable after adaptation	(b)	110	13.4	3.2	
Increase of viable during adaptation	(c)	19	4.3	2.6	
Velocity of galactose fermentation, μ l. $\text{C}_6\text{H}_{12}\text{O}_6$ /hr.	(R)	200	198	127	
	(R/a)	2.3	21.7	215	
	(R/b)	1.9	14.8	39.6	
	(R/c)	11	46	49	

Cells/ml. $\times 10^6$	Exp. 2	Time of exposure (min.)			
		0	3	6	10
Total before adaptation		147	—	—	—
Viable before adaptation	(a)	8.4	5.3	0.165	0.000036
Viable after adaptation	(b)	55	29.5	2.57	0.00085
Increase of viable during adaptation	(c)	47	24.2	2.41	0.00081
Velocity of galactose fermentation, μ l. CO_2 /hr.	(R)	243	215	51	0
	(R/a)	29	40.6	315	—
	(R/b)	4.4	7.3	19.9	—
	(R/c)	5.2	8.9	24	—

Though we do not advance these experiments as proof that adaptation is independent of viability, we think the variability of the ratios (R/a , R/b and R/c) strongly suggests this conclusion. Nevertheless this variability is of a totally different order from that obtained in Table IV, where viability and enzyme activity are in question and where indeed the ratio viable cells/enzyme activity varies from 1.98×10^{-4} to ∞ .

Non-adaptive nature of glucozymase.

Since yeast is always grown on media containing glucose or sugars giving glucose on hydrolysis it seemed possible that glucozymase itself might be adaptive. *S. cerevisiae* was accordingly grown for four subcultivations on broth with purified galactose free from glucose. At the end its fermentative power on glucose was unimpaired, hence glucozymase in this strain is not an adaptive enzyme.

Influence of glucose on galactozymase.

Owing to conflicting reports in the literature, we investigated the influence of glucose on galactozymase. Tubes of yeast were adapted in galactose and the

fermentation rate determined on successive days. On the second day 6 tubes were centrifuged and the yeast washed and replaced as follows:

- 2 with fresh galactose;
- 2 with water;
- 2 with glucose.

The fermentation rates of all were determined on the two successive days; the results are shown in Fig. 3. It is seen that the replacement of the galactose by glucose in the training tubes causes a disappearance of galactozymase which is almost complete in 2 days. Fresh galactose, on the contrary, causes a slight increase in galactozymase, which again falls off, whilst, when galactose is replaced

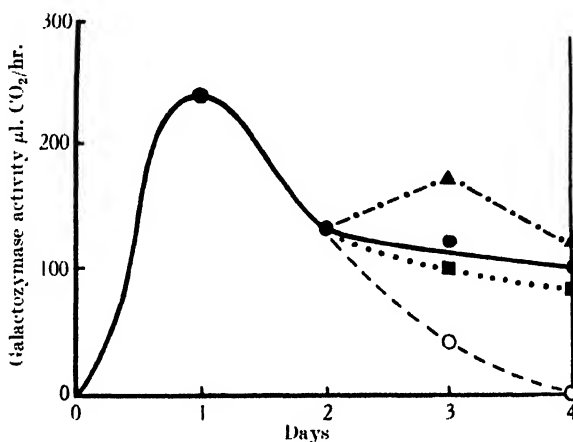


Fig. 3. ●—● Adaptation in galactose; ▲-.-▲ fresh galactose added; ■...■ galactose replaced by water; ○- -○ galactose replaced by glucose.

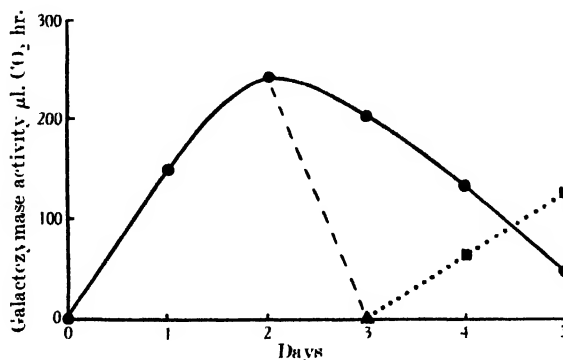


Fig. 4. ●—● Adaptation in galactose; ●- -● replacement of galactose by glucose; ■...■ replacement of glucose by galactose.

by water, the course of the fermentation closely follows the course of the tubes where the original galactose is undisturbed. Fig. 4 gives a similar experiment showing a disappearance of galactozymase on treatment of the yeast with glucose and its reappearance on the renewal of galactose.

SUMMARY.

1. In confirmation of Dienert and of others we find that in *S. cerevisiae* galactozymase is an adaptive enzyme.

2. In confirmation of Dienert and of Abderhalden adaptation was proved to occur in aqueous solutions of galactose.

3. Adaptation can occur without cell multiplication. Thus, as in the case of formic hydrogenlyase, adaptation occurs, not as a result of natural selection, but as a response of the cell to its chemical environment.

4. We have so far been unable to obtain adaptation in sterile cultures, although a study of total and viable counts suggests that it is not viable cells alone which are capable of adaptation.

5. The activity of galactozymase and of glucozymase is independent of cell viability.

6. In confirmation and extension of earlier work we find that adapted cells lose their galactozymase completely after fermenting glucose and regain it in the presence of galactose.

We take pleasure in thanking Sir Frederick Hopkins for the interest he has shown in this investigation.

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LXXVII. HYDROGENLYASES.

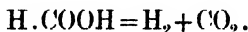
IV. THE SYNTHESIS OF FORMIC ACID BY BACTERIA.

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THE formic hydrogenlyase enzyme of bacteria has been studied in detail by Stephenson and Stickland [1932; 1933] and by Yudkin [1932]. This enzyme catalyses the reaction:



Recent work has shown the importance of H_2 and CO_2 in reactions induced by bacteria [Gaffron, 1935; Roelofsen, 1934; Kubowitz, 1934]. It was therefore of interest to determine whether the formic hydrogenlyase system could be reversed. Stephenson and Stickland [1932] found a quantitative formation of H_2 from formic acid, but since, in their experiments, the other reaction product, carbon dioxide, was absorbed, an equilibrium was scarcely to be expected. An early observation of Harden [1901] indicates the possibility of such an equilibrium. In the fermentation of glucose by *Bact. coli* he found that an increase of pressure reduced the production of H_2 and CO_2 and increased the production of formic acid.

In the experiments to be described it will be shown that the formic hydrogenlyase system can be reversed, and that if washed suspensions of *Bact. coli* and other organisms are shaken in a gas mixture of H_2 and CO_2 , gas is taken up and formic acid is produced.

Preparation of bacterial suspensions.

The cultures were obtained from the National Collection of Type Cultures. *Bact. coli* (Escherich) was used for most of the work. It was grown under the optimum conditions for the formation of the formic hydrogenlyase enzyme [Yudkin, 1932]. Tryptic caseinogen broth containing 0.5 % formic acid as sodium formate was sown from a 12-hour culture of *Bact. coli* on broth. After 14 hours' growth the organism was centrifuged down and washed twice on the centrifuge with distilled water. The growth from 1 l. of medium was usually finally suspended in 10 ml. water. Such a suspension averaged 0.8 ± 0.2 mg. N/ml. (6.6 ± 1.6 mg. dry weight/ml.).

Measurement of gas absorption.

Gas changes were measured in Warburg manometers. The cups contained bacterial suspension and NaHCO_3 of final concentration 0.025 *M*. The manometers were filled with a gas mixture of 5 % CO_2 in H_2 ; the p_{H} was 7.4. Controls were done with 5 % CO_2 in N_2 and with H_2 (using phosphate buffer). Conical cups with side-bulbs and inner cups, total volume 18–20 ml., were used for most of the work.

Gas changes will usually be expressed as Q values ($\mu\text{l.}/\text{hour}/\text{mg. dry weight}$). The total nitrogen of the suspensions was determined and converted to dry weight by use of the figures for N % of dry weight given by Yudkin [1932].

It will be realised that if H_2 and CO_2 combine with production of formic acid the only gas change to be measured in the manometers would be the H_2 uptake; the production of one equivalent of formic acid from one equivalent of CO_2 would liberate again one equivalent of CO_2 from the bicarbonate buffer.

The manometers were equilibrated in the bath for 20 min. before the initial readings were taken. All experiments were done at 38° unless otherwise stated.

EXPERIMENTAL.

Table I gives the results of an experiment designed to show the gas uptake from a H_2 - CO_2 mixture. It will be seen that there is a considerable gas uptake from H_2 - CO_2 whilst the blanks in N_2 - CO_2 and in H_2 are negligible.

Table I.

Gas used	5% CO_2 in H_2	5% CO_2 in N_2	H_2
<i>Bact. coli</i> suspension (ml.)			0.5	0.5	0.5
0.125 <i>M</i> NaHCO_3 (ml.)			0.6	0.6	—
0.2 <i>M</i> phosphate p_{H} 7.4 (ml.)			—	—	0.6
Water (ml.)			1.9	1.9	1.9
mm. Brodie's solution/hour			- 200	1	- 6
$\mu\text{l. H}_2/\text{hour}$			- 256	.	- 8
Q_{H_2}			- 76	-	- 2

It is now necessary to establish that the only gas change occurring is an uptake of H_2 , for, as has been pointed out, if formic acid is the product the CO_2 uptake would be balanced by a corresponding evolution of CO_2 from the bicarbonate buffer. Use was made of the principle of Warburg's two vessel method. The gas uptake was measured using small and large volumes of fluid in the cups. (The volume of the cups was 10–12 ml.) If the only gas uptake is H_2 then $\mu\text{l.}$ gas change per ml. bacterial suspension should be the same; if CO_2 is also absorbed, then owing to the relatively high solubility of CO_2 the measured gas uptakes in the two cases will differ. The results of such experiments are given in Table II.

Table II.

	Exp. 1		Exp. 2	
<i>Bact. coli</i> suspension (ml.)	2	5	2	5
$\mu\text{l. H}_2/35 \text{ min.}$	- 31.1	- 75.2	- 22.7	- 55.7
$\mu\text{l. H}_2/35 \text{ min.}/\text{ml. suspension}$	- 15.5	- 15.1	- 11.3	- 11.1
Q_{H_2}	- 71	- 69	- 52	- 51

A temperature of 25° was used in this case since the uptake of gas is more constant; at 38° the uptake in the cups containing the larger volume of fluid fell off rather rapidly. 1 ml. of *M* NaHCO_3 solution was diluted with 22 ml. water and the mixture equilibrated with the H_2 - CO_2 gas mixture for 30 min. at 25° . 2 ml. *Bact. coli* suspension were then added. The resulting suspension was measured into the manometer cups and the manometers filled with gas. Readings were commenced after a further 20 min. equilibration in the bath, when the uptake had become steady, and were taken at 5-min. intervals. The results show sufficient agreement to justify the conclusion that H_2 uptake is the only gas change measured.

It may be mentioned at this point that the Q_{H_2} values obtained with various batches of *Bact. coli* varied from 50 to 90. Further, the activity fell off fairly rapidly with time, the Q_{H_2} value being halved on standing 24 hours at 0°.

If formic acid is produced and liberates from the bicarbonate present a quantity of CO_2 equal to that absorbed during its synthesis, then there should be a disappearance of bicarbonate from the system. This was demonstrated in the following experiment. Warburg cups contained 0.5 ml. *Bact. coli* suspension, 0.6 ml. 0.031 *M* NaHCO_3 , 0.7 ml. water and 0.2 ml. 2*N* H_2SO_4 (in the side-bulb). The manometers were filled with the usual 5% CO_2 in H_2 mixture. After equilibration the acid in one set of manometers was tipped into the main cup and the evolution of CO_2 measured. This gave the amount of bicarbonate present at the beginning of the experiment. Meanwhile, in the second series of manometers, the uptake of H_2 was measured for 80 min. At the end of this period the acid was tipped into the main cup, the CO_2 output giving in this case a measure of the bicarbonate finally present. The tipping in of the acid is, of course, sufficient to stop any enzyme action. The results of two experiments are given in Table III.

Table III.

	Exp. 1	Exp. 2
Initial bicarbonate ($\mu\text{l. CO}_2$)	364	364
Final bicarbonate ($\mu\text{l. CO}_2$)	230	228
Bicarbonate used ($\mu\text{l. CO}_2$)	134	136
H_2 uptake ($\mu\text{l. H}_2$)	117	126
H_2/CO_2	1.13	1.07

It is thus established that bicarbonate, as well as H_2 , disappears from the system and further that these disappear in approximately equimolecular proportions. The experiments summarised in Table II also show that for each molecule of H_2 absorbed one molecule of a monobasic acid is formed (or a half molecule of a dibasic acid and so on).

Detection of formic acid.

One method for the detection and estimation of formic acid depends on the fact that it is a volatile acid and can reduce mercuric chloride to mercurous chloride [Dakin, 1913]. The growth of *Bact. coli* from 1 l. of broth-formate medium was finally suspended in 15 ml. water. To 10 ml. of this suspension were added 1 ml. *M* NaHCO_3 and 14 ml. water (A). The other 5 ml. were diluted with 1 ml. 0.5 *M* phosphate buffer p_{H} 7.4 and 6.5 ml. water (B). 10 ml. of (A) were placed in a 75 ml. pot and the pot filled with the 5% CO_2 in H_2 gas mixture and shaken for 6 hours at 38°. Controls were done with 10 ml. (A) in 5% CO_2 in N_2 and with 10 ml. (B) in H_2 alone. The contents of each pot were then treated as follows. 1 ml. of phosphoric acid was added and the acid mixture distilled *in vacuo* almost to dryness, the distillate being collected under 5 ml. of 0.1 *N* NaOH . 25 ml. of 50% alcohol were added and the distillation repeated. The distillate was evaporated to 10 ml. on the water-bath, acidified with 5 ml. of 5% acetic acid (formate-free) and 40 ml. of a saturated solution of mercuric chloride added. This mixture was heated in a sealed flask on the boiling-water bath for 6 hours. The precipitated calomel was filtered off through a weighed Gooch crucible, washed with 5% HCl , dried to constant weight at 90°, and weighed. The results of a typical experiment are given in Table IV and indicate clearly that the product is a volatile acid which can reduce mercuric chloride to calomel. Formic acid is the only common volatile acid with this property. The next step is to show that, assuming the acid to be formic acid, the total volatile acid produced is quantitatively equal to the formic acid as estimated by the calomel method. For this

Table IV.

Gas mixture	5% CO ₂ in H ₂	5% CO ₂ in N ₂	H ₂
Bacterial suspension (ml.)			10 (A)	10 (A)	10 (B)
Calomel formed (mg.)			54.6	1.2	0.5
Formic acid (mg.)			5.33	0.11	0.05

purpose it is necessary to obtain a larger amount of product. The growth of *Bact. coli* from 2 l. of broth-formate medium was suspended in 60 ml. 0.04 *M* NaHCO₃ and a stream of the 5% CO₂ in H₂ mixture passed through for 10 hours at 38°. The suspension was then acidified and the volatile acid estimated by a method described for acetic acid by Stephenson and Whetham [1922]. The neutralised distillates were evaporated and the formic acid estimated in duplicate by the calomel method. The results, given in Table V, show that the total volatile acid is in good agreement with the formic acid as estimated by its reducing property.

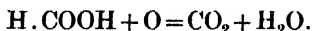
Table V.

Gas mixture	Volatile acid method		Calomel method	
	Volatile acid, ml. 0.1 <i>M</i>	H.CO.OH equivalent, mg.	H.CO.OH, mg.	Volatile acid equivalent, ml. 0.1 <i>M</i>
5% CO ₂ in H ₂	8.77	—	37.8	—
5% CO ₂ in N ₂	0.23	—	0.4	—
Difference	8.54	39.2	37.4	8.13

H.CO.OH (by calomel)/H.CO.OH (by titration) = 95.2%.

Identification of formic acid by biological methods.

Further evidence that the product of the reduction of CO₂ by H₂ in the presence of *Bact. coli* is formic acid is provided by the decomposition of the product by *Bact. coli* itself. Anaerobically in N₂ the formic hydrogenlyase enzyme decomposes formic acid to H₂ and CO₂, and, if the CO₂ is absorbed, H₂ is produced quantitatively [Stephenson and Stickland, 1932]. Aerobically formic acid is decomposed by *Bact. coli*:



The oxygen uptake is theoretical [Cook and Stephenson, 1928] and may be measured if the CO₂ is absorbed.

The acid was prepared on a large scale as already described, distilled into dilute NaOH, evaporated, carefully neutralised and made up to 10 ml. Formic acid was estimated in duplicate on 2 ml. portions by the calomel method. For the anaerobic decomposition the Warburg cups contained 0.5 ml. *Bact. coli* suspension, 1 ml. phosphate buffer *p*_H 7.4, 0.2 ml. of the distillate (in the side-bulb) and filter-paper rolls moistened with 0.2 ml. of 10% KOH in the centre-tubes for CO₂ absorption. Controls were done with 0.2 ml. formate of known strength (approximately equal to that of the distillate) and with 0.2 ml. water. The manometers were filled with N₂. After equilibration the substrate was tipped in from the side-bulb and the gas evolution measured to completion. The aerobic decomposition experiment was performed in the same way except that the manometers contained air and the oxygen uptake was measured. The results of an experiment are given in Table VI and provide excellent quantitative evidence that the distillate contains formic acid. The slightly over-theoretical oxygen uptake has always been observed and no explanation can be offered for this.

Table VI.

0.2 ml. distillate (0.538 mg. H.COOH by calomel method)						0.2 ml. 0.05 <i>M</i> H.COONa (0.46 mg. H.COOH)					
H ₂ evolved (μl.)			O ₂ uptake (μl.)			H ₂ evolved (μl.)			O ₂ uptake (μl.)		
Theo- retical	Found	%	Theo- retical	Found	%	Theo- retical	Found	%	Theo- retical	Found	%
262	249	95	131	144	110	224	216	96	112	123	110

Further evidence that the distillate contains formic acid is provided by its behaviour with a formic dehydrogenase preparation from *Bact. coli*. This preparation [Stickland, 1929] is highly specific for formic acid; traces only of lactic and succinic dehydrogenases may be present. Thunberg tubes were set up containing 1 ml. phosphate buffer p_H 7.4, 1 ml. 1 : 5000 methylene blue, 1 ml. of a suitable dilution of the enzyme preparation and 1 ml. of the substrate. Distillate containing approximately *M*/200 formic acid (by the calomel method) was used. The tubes were evacuated and the time taken for the reduction of the methylene blue noted. The results are given in Table VII and show that the

Table VII.

Substrate	Reduction time
Water blank	> 6 hours
Formate <i>M</i> /200	2 min. 30 sec.
Lactate <i>M</i> /200	> 6 hours
Succinate <i>M</i> /200	> 6 hours
Distillate	2 min. 30 sec.

distillate contains formic acid. Owing to the very high affinity of the formic dehydrogenase enzyme for its substrate a quantitative estimation of formic acid by this method is not possible. A rough idea may be obtained by running parallel dilutions of the distillate and of formate of known concentration. Some results are given in Table VIII and show the concentration of formic acid as estimated by this method and by the calomel method to be of the same order.

Table VIII.

Dilution	Reduction times	
	Distillate (<i>M</i> /15 formate by calomel method)	Formate <i>M</i> /15
	min.	min.
1 : 20	5	4.5
1 : 80	7	6.75
1 : 160	9	9
1 : 320	50% reduced only	50% reduced only

The evidence that the product of the reduction of CO₂ by H₂ in the presence of washed suspensions of *Bact. coli* is formic acid may be summarised as follows:

- (1) The manometric data agree with this assumption.
- (2) It is a volatile acid.
- (3) It reduces mercuric chloride.
- (4) The H₂ formed when it is decomposed by *Bact. coli* anaerobically, and the oxygen uptake when oxidised aerobically agree exactly with the values obtained for formic acid by other methods.
- (5) It reduces methylene blue in the presence of the specific formic dehydrogenase preparation.

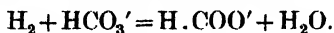
Quantitative relation between H₂ uptake and formic acid produced.

The H₂ uptake of *Bact. coli* suspension from 5 % CO₂ in H₂ gas mixture was measured in a series of eight manometers, the bacterial suspension being tipped in from the side-bulb after equilibration and readings started at once. At the end of the experiment the contents of the cups were acidified with phosphoric acid and the total formic acid content estimated by the calomel method in the usual way. The results of two experiments are shown in Table IX.

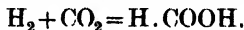
Table IX.

H ₂ uptake μl.	H.CO ₂ H calc. mg.	H.CO ₂ H found mg.	% found of calc.
2931	6.02	5.79	96
2803	5.76	5.58	97

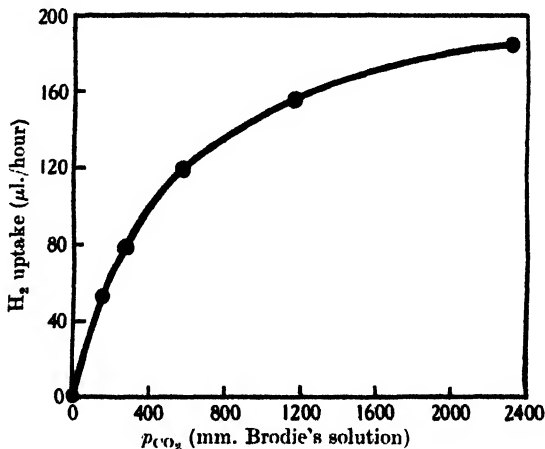
Since it has already been shown that the ratio H₂/HCO₃' is 1, the reaction is proved to be, under the actual experimental conditions:



Nothing definite can be said, however, as to the possibility of the primary reaction being:

*Effect of the partial pressure of carbon dioxide.*

For these experiments the manometers were filled with H₂ and varying pressures of CO₂ generated in the cup by mixing NaHCO₃ with acid. The final concentration of NaHCO₃ was also varied with the pressure of CO₂ in order to maintain a constant p_{H} . The effect may also be due, therefore, to the increase in NaHCO₃ concentration. The partial pressure of H₂ decreases proportionally. The results are plotted in Fig. 1 and show that the H₂ uptake increases with increasing partial pressure of CO₂ up to about 0.25 atmosphere.

Fig. 1. Effect of partial pressure of CO₂ (1 atmosphere – 10,000 mm. Brodie's solution).*Effect of bicarbonate concentration.*

The results of an experiment in which various concentrations of bicarbonate were used with the usual 5 % CO₂ in H₂ gas mixture are shown in Fig. 2. The p_{H} also varies with the bicarbonate concentration, so that the net effect is probably due in part to the influence of p_{H} and in part to the concentration of bicarbonate.

In the case of the lower concentrations of bicarbonate the falling off of the rate of gas uptake with time is due to the exhaustion of bicarbonate (which disappears during the reaction). At the highest bicarbonate concentration, 0.1 *M*, the slow rate is no doubt due to the high p_{H} of 8.1.

Experiments with bicarbonate present, but not CO_2 (in H_2 alone), and with CO_2 but no bicarbonate (at acid p_{H} values), gave no H_2 uptake. It is impossible to dissociate these results from possible p_{H} effects. It can be said that in the actual conditions when H_2 is absorbed both CO_2 and bicarbonate are present, and that the rate of absorption seems to depend on the concentration of both.

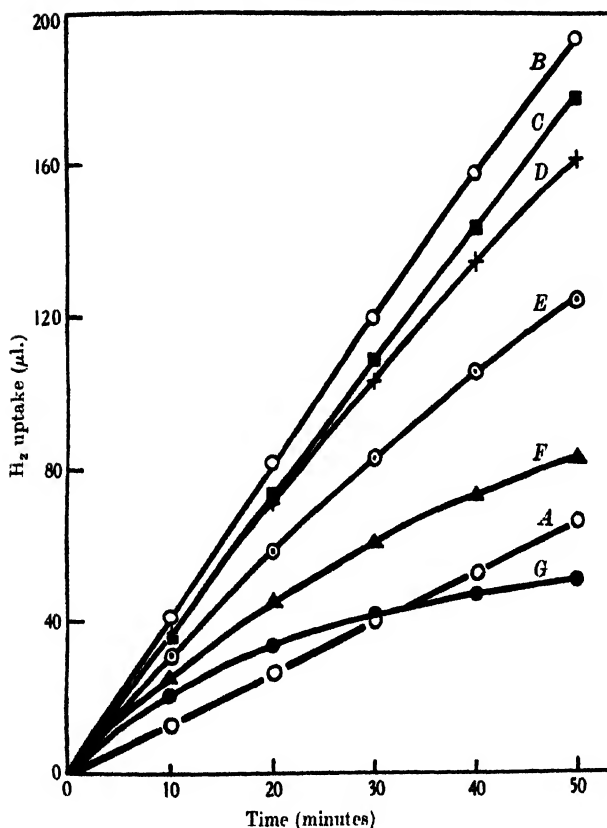


Fig. 2.

Fig. 2. Effect of bicarbonate concentration.

- | | |
|---|--|
| A. —○— $M/10 \text{ NaHCO}_3$; p_{H} 8.1. | E. —○— $M/160 \text{ NaHCO}_3$; p_{H} 6.9. |
| B. —○— $M/20 \text{ NaHCO}_3$; p_{H} 7.8. | F. —▲— $M/320 \text{ NaHCO}_3$; p_{H} 6.6. |
| C. —■— $M/40 \text{ NaHCO}_3$; p_{H} 7.5. | G. —●— $M/640 \text{ NaHCO}_3$; p_{H} 6.9. |
| D. —x— $M/80 \text{ NaHCO}_3$; p_{H} 7.2. | |

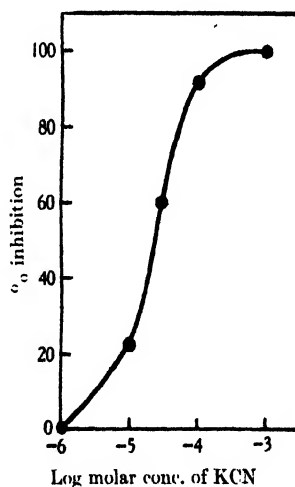


Fig. 3.

Fig. 3. Effect of KCN.

Effect of poisons.

The system was found to be sensitive to the same poisons as the reverse reaction studied by Stephenson and Stickland [1932]. It was completely inhibited by toluene, chloroform and 1 % sodium fluoride. The effect of KCN is shown in Fig. 3.

Effect of temperature.

The Q_{H_2} for the reaction was measured at 38° and at 20° with the same bacterial suspension. The Q_{H_2} at 38° was -71 and at 20° -51. The decrease of the rate of reaction with temperature is less than usually found in reactions brought about by *Bact. coli*; the effect of temperature is offset to some extent in this case by the mass effect due to the increased solubility of CO_2 at the lower temperature.

Effect of growth conditions.

Stephenson and Stickland [1932] and Yudkin [1932] found that washed suspensions of *Bact. coli* contained the enzyme formic hydrogenlyase only when the organism was grown in a medium containing formate or substances giving rise to formate by the action of the bacterium. The results summarised in Table X show that, as far as the matter was studied, this is the case also for the

Table X.

Growth medium	Q_{H_2} values	
	Synthesis of formate	Decomposition of formate
Broth-formate	- 70	91
Broth	- 26	35
Broth agar (Roux)	- 1	1
Extracted broth-formate	- 67	93
Extracted broth	- 6	10

reverse system dealt with in this paper. As the stock tryptic broth was found to contain small amounts of formic acid it was extracted at p_{H_2} 2 with ether for 72 hours. After this time the ether extract contained no formate. For comparison Q_{H_2} values for the decomposition of formate (measured in N_2 - CO_2 mixture, formate 0.03 *M* in bicarbonate buffer) are also given. The experiments with extracted broth clearly show the adaptive nature of the enzyme. The Q_{H_2} values for the synthesis and for the splitting of formate run parallel, this is to be expected if the same enzyme is responsible for both reactions.

Distribution of the enzyme.

Yudkin [1932] studied the distribution of the formic hydrogenlyase enzyme in some detail. The distribution of the power to induce the reverse reaction, in so far as it was studied, was found to be similar in every way to that of the formic hydrogenlyase enzyme (Table XI).

Table XI.

Species	Growth medium	Q_{H_2} values	
		Synthesis of formate	Decomposition of formate
<i>Bact. coli</i> (Esch.)	Broth-formate	- 70	91
<i>Bact. coli</i> (3813)	Broth-formate	- 63	89
<i>Bact. lactis aerogenes</i> (124)	Broth-formate	- 51	64
	Broth agar (Roux)	0	0
<i>Bact. dispar</i>	Broth-formate	0	0
<i>Bact. freundii</i>	Broth-formate	- 75	83
<i>Bact. cloacae</i>	Broth	0	0
	Broth-formate	0	0
	Broth-glucose	- 26	56

Comparison of Tables X and XI with Table II of Stephenson and Stickland [1932] also shows that the presence of the formic hydrogenlyase enzyme is essential if the synthesis is to occur.

Determination of the equilibrium point.

The concentration of formate in equilibrium with a given $\text{H}_2\text{-CO}_2$ gas mixture in the presence of *Bact. coli* was determined in the following experiments. A preliminary experiment showed the equilibrium concentration of formate to lie between 0.1 *M* and 0.01 *M*. Manometers were set up containing NaHCO_3 in final concentration of 0.025 *M* and formate in various final concentrations between 0.1 *M* and 0.0125 *M*. *Bact. coli* suspension was placed in the side-bulbs and the manometers were filled with the 5% CO_2 in H_2 mixture. After equilibration the bacterial suspension was tipped in and the initial rates of H_2 uptake or evolution measured (in order that the concentration of bicarbonate should not decrease appreciably). The experiments were done both at 25° and at 38°. The initial rate of evolution or absorption of H_2 , less the controls in H_2 and in $\text{N}_2\text{-CO}_2$, were plotted against the logarithm of the formate concentration. The mean values from a number of experiments are plotted in Fig. 4. From these curves

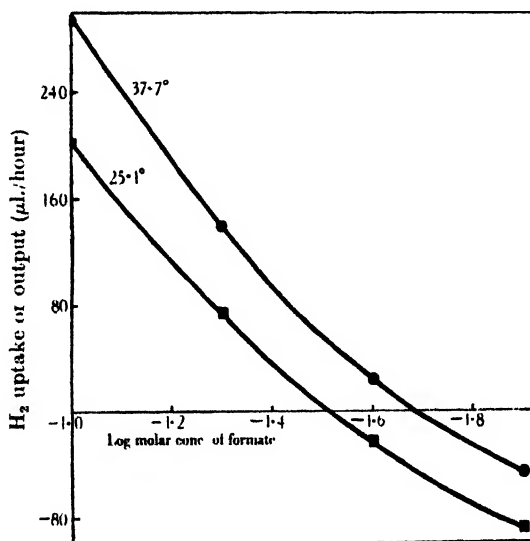


Fig. 4. Determination of equilibrium point.

the concentration of formate at which the gas exchange is zero may be obtained. This point, at which H_2 is being absorbed at the same rate as it is evolved, gives the equilibrium concentration of formate in the given experimental conditions. These values, together with other experimental data to be used in the next section, are given in Table XII.

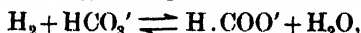
Table XII.

	Series 1	Series 2
Temperature (° absolute, ° K.)	298.1	310.7
Formate concentration (<i>M</i>)	0.0306	0.0204
Bicarbonate concentration (<i>M</i>)	0.025	0.025
Partial pressure of H_2 (atmos.)	0.916	0.884
(corrected for atmospheric pressure and pressure of water vapour)		

Calculation of the free energy of the reaction.

From the equilibrium concentrations of the reactants it is possible to calculate the free energy change of the reaction. It is also possible, from the known free energy values of the reactants, to compute the theoretical free energy change. If the equilibrium measured is a real one these two values should agree within the limits of the errors involved.

Calculation has been made by the conventional procedures of Lewis and Randall [1923]. The free energy change at the standard state¹ ($\Delta F_{\text{obs.}}$) for the reaction,



is related to the activities of the reactants ($[\text{HCOO}']$, etc.) as follows:

$$\Delta F_{\text{obs.}} = -RT \ln K_{\text{obs.}} = -RT \ln \left(\frac{[\text{H.CO.O}'] [\text{H}_2\text{O}]}{[\text{HCO}_3'] [\text{H}_2]} \right),$$

where $K_{\text{obs.}}$ is the equilibrium constant.

Following the usual conventions, the activity of hydrogen is taken as its partial pressure and the activity of water as its mol. fraction (calculated as 0.998 at the experimental conditions); the activities of the anions are expressed as molal activities ($\gamma \times \text{molal concentration}$). The activity coefficient (γ) of HCO_3' was calculated from the ionic strength as described by Hastings and Sendroy [1925], and equalled 0.7615 at the equilibrium point. As no value could be found in the literature for the activity of HCOO' , its activity coefficient was taken as equal to that of HCO_3' at the ionic strength in question. Then taking the experimental data of the last section referring to 298.1° K.,

$$\Delta F_{\text{obs.}} = -RT \ln \left(\frac{0.0306 \times 0.7615 \times 0.998}{0.025 \times 0.7615 \times 0.916} \right) = -171 \text{ calories.}$$

The theoretical free energy change of the reaction at the standard state will now be calculated from known free energy values. The data used were: free energy of H.CO.OH (aq. 1 mol.) = -87,590 cal. [Kay and Parks, 1934]; free energy of water = -56,720 cal. [Giauque and Ashley, 1933]; free energy of CO_2 (g. 1 atm.) = -94,443 cal. [Gordon, 1933]; ionisation constant of H.CO.OH = 1.772×10^{-4} [Harned, 1934]; saturated solution of CO_2 = 0.03353 *M* [MacInnes and Belcher, 1933]; first ionisation constant of H_2CO_3 , $p_K = 6.3655$ [Shedlovsky and MacInnes, 1935]. All these values are for 298° K. No values for the ionisation constants of the two acids or for the solubility of CO_2 appear to be available for the ionic strength existing at the standard state; this may be a source of error in the following calculations.

If the free energy of H_2 be represented as F_{H_2} and so on, then for the reaction:



$$\Delta F_0 = (F_{\text{HCOO}'} + F_{\text{H}_2\text{O}}) - (F_{\text{H}_2} + F_{\text{HCO}_3'}) \quad \dots\dots(1),$$

if ΔF_0 is the free energy change of the reaction.

Computation of $F_{\text{H.CO.O}'}$. If K_1 is the ionisation constant of H.CO.OH then, free energy of ionisation of H.CO.OH = $-RT \ln K_1 = +5119$ cal.

from which since

$$F_{\text{H.CO.O}'} = F_{\text{H.CO.OH}} + F_{\text{of ionisation}},$$

$$F_{\text{H.CO.O}'} = -82,471 \text{ cal.}$$

Computation of $F_{\text{HCO}_3'}$. If α is the molal solubility coefficient of CO_2 then,

$$\text{free energy of solution of } \text{CO}_2 = -RT \ln \alpha = +2013 \text{ cal.,}$$

also

$$F_{\text{CO}_2 \text{ (aq. sat.)}} = F_{\text{CO}_2 \text{ (g.)}} + F_{\text{of solution}} = -92,430 \text{ cal.}$$

¹ The standard state is defined as 1 molal activity in the case of the solutes, 1 atmos. pressure in the case of H_2 and a mol. fraction of unity in the case of water.

Assuming the activity coefficient of CO_2 (aq.) to be 1, the free energy change of converting CO_2 (aq. sat.) to CO_2 (1 molal activity) = $-RT \ln \frac{0.03353}{1} = +2013$ cals., from which, since

$$F_{\text{CO}_2} \text{ (aq. 1 molal)} = F_{\text{CO}_2} \text{ (aq. sat.)} + F_{\text{of concentration}},$$

$$F_{\text{CO}_2} \text{ (aq. 1 molal)} = -90,417 \text{ cals.}$$

Therefore

$$F_{\text{H}_2\text{CO}_3} = F_{\text{CO}_2} + F_{\text{H}_2\text{O}} = -147,137 \text{ cals.}$$

If K_1' is the first ionisation constant of H_2CO_3 , then, free energy of ionisation of $\text{H}_2\text{CO}_3 = -RT \ln K_1' = +8688$ cals., so that

$$F_{\text{HCO}_3'} = F_{\text{H}_2\text{CO}_3} + F_{\text{of ionisation}} = -138,449 \text{ cals.}$$

Now since $F_{\text{H}_2} = 0$ and $F_{\text{H}_2\text{O}} = -56,720$ cals., substituting these values and those calculated above for $F_{\text{HCOO}'}$ and $F_{\text{HCO}_3'}$ in equation (1) we find

$$\Delta F_0 = -742 \text{ cals.}$$

This value differs by 571 cals. from the value of 171 cals. obtained for ΔF_{obs} . The agreement is very satisfactory and is well within the order of accuracy usually found in such calculations. It may be noted that according to Parks and Huffman [1932] the free energy of formic acid may be in error ± 300 cals., whilst the latest value for the free energy of CO_2 taken above varies by 300 cals. from earlier values. The ionisation constants of the two acids have been revised considerably in recent years and may be subject to further revision. Other possible sources of error have already been pointed out.

Calculation of the heat of the reaction.

From the equilibrium constants at two temperatures it is possible, by means of the Van't Hoff isochor, to calculate the heat of the reaction. This should agree with the heat of the reaction computed from known thermal data if the equilibria measured are real. Using the results of the equilibrium experiments at 298.1°K . and 310.7°K ., the equilibrium constants were calculated as in the last section. Then if K_T and K_{T_1} be these constants for 310.7°K . and 298.1°K . respectively,

$$\frac{K_T}{K_{T_1}} = \frac{0.0204 \times 0.916}{0.0306 \times 0.884}.$$

From the Van't Hoff isochor

$$\Delta H_{\text{obs.}} = \frac{R(TT_1)}{T - T_1} \ln \frac{K_T}{K_{T_1}},$$

where $\Delta H_{\text{obs.}}$ is the heat of the reaction and T and T_1 are the absolute temperatures corresponding to K_T and K_{T_1} , from which, $\Delta H_{\text{obs.}} = -5407$ cals.

Calculation of ΔH from known thermal data. The following ΔH values were used; all were obtained from Parks and Huffman [1932]:

$$\text{H.COOH (l), } \Delta H = -99,750 \text{ cals.;}$$

$$\text{CO}_2 \text{ (g), } \Delta H = -94,240 \text{ cals.;}$$

$$\text{H}_2\text{O (l), } \Delta H = -68,310 \text{ cals.}$$

All are for 298°K .

Then since $\text{CO}_2 \text{ (g)} \rightleftharpoons \text{CO}_2 \text{ (sat. aq.)} + 4844$ cals. [Adolph and Henderson, 1922],

$$\Delta H \text{ of } \text{CO}_2 \text{ (sat. aq.)} = -94,240 - 4844 = -99,084 \text{ cals.}$$

So that

$$\Delta H \text{ of } \text{H}_2\text{CO}_3 = \Delta H \text{ of } \text{CO}_2 \text{ (aq.)} + \Delta H \text{ of } \text{H}_2\text{O} = -167,394 \text{ cals.}$$

Also $\text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3' + \text{H}^+ - 2075$ cals. [Shedlovsky and MacInnes, 1935], from which,

$$\Delta H \text{ of } \text{HCO}_3' = -167,394 + 2075 = -165,319 \text{ cals.}$$

According to Parks and Huffman [1932],

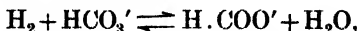


therefore, ΔH of $\text{HCOOH (aq.)} = -99,750 - 100 = -99,850 \text{ cal.},$

and since $\text{H.COOH (aq.)} \rightleftharpoons \text{H.COO'} + \text{H'} + 13 \text{ cal. [Harned, 1934],}$

$$\Delta H \text{ of } \text{H.COO'} = -99,850 - 13 = -99,863 \text{ cal.}$$

If ΔH_0 is the heat of the reaction:



$$\begin{aligned} \Delta H_0 &= (\Delta H_{\text{H.COO'}} + \Delta H_{\text{H}_2\text{O}}) - (\Delta H_{\text{H}_2} + \Delta H_{\text{HCO}_3'}) \\ &= -2854 \text{ cal.} \end{aligned}$$

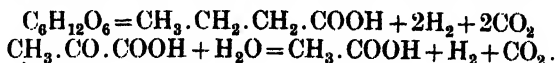
This is in reasonable accord with the value of -5407 cal. calculated from the observed equilibria; an agreement of $\pm 3000 \text{ cal.}$ is usually considered satisfactory in such calculations. The main source of error probably lies in the fact that the temperature difference of 12.6° is rather large: the calculation from thermal data is for 298° K. , no values for higher temperatures being available.

DISCUSSION.

It has been shown that the distribution of the enzyme system studied in this paper is identical with that of the formic hydrogenlyase system of Stephenson and Stickland. Further it has been demonstrated that the presence in the washed cells of the synthesising enzyme is conditioned, as with formic hydrogenlyase, by the presence of formate in the culture medium. Both systems are sensitive to the same poisons. It seems justifiable to conclude, therefore, that the same enzyme catalyses both the synthesis and the breakdown of formic acid, that is, that the formic hydrogenlyase enzyme system is reversible. This view is confirmed by the free energy and heat calculations for the reaction. The values calculated from the observed equilibrium agree (within the limits of the accuracy of the data available and the experimental methods) with the values computed from known thermodynamical data, indicating that the observed equilibrium is a real one.

The reversal of the formic hydrogenlyase system may provide a satisfactory explanation for several cases recorded in the literature of inhibition by hydrogen of bacterial reactions producing hydrogen and carbon dioxide. Stephenson and Stickland [1932] found that the breakdown of formate by the formic hydrogenlyase enzyme was inhibited by cylinder hydrogen to the extent of 40%. Since hydrogen from a Kipp's apparatus completely inhibited the reaction they thought that the effect might be due in both cases to impurities in the gas. It now seems possible that the reverse reaction was in fact occurring, for some carbon dioxide would remain in solution as bicarbonate (at $p_{\text{H}} 7$) in spite of the NaOH papers used in their experiments to absorb carbon dioxide.

Kubowitz [1934] found that hydrogen, as compared with argon, partially inhibited the gas production of certain reactions brought about by *Cl. butyricum*:



In argon the concentration of hydrogen produced would be too small for a reverse reaction between hydrogen and carbon dioxide to be of quantitative importance; in an atmosphere of hydrogen, however, a synthesis of formic acid may well explain the low values obtained by Kubowitz.

It is also conceivable that a synthesis of formic acid similar to that described in this paper may help to explain the differences between the work of Gaffron [1935] and that of Roelofsen [1934] concerning the reduction of carbon dioxide by hydrogen in the dark in the presence of the red sulphur bacteria. Gaffron has already suggested that Roelofsen's results were due to an inhibition of carbon dioxide production by hydrogen, quoting the cases mentioned above as examples of such an inhibitory effect of hydrogen on bacterial fermentations.

SUMMARY.

The formic hydrogenlyase enzyme system of bacteria has been shown to be reversible.

I wish to express my thanks to Miss M. Stephenson for suggesting this research and for her criticism and advice throughout. I am deeply indebted to Dr D. E. Green for help with the free energy calculations, and to Sir F. G. Hopkins for his interest in this work. The preliminary experiments were carried out in collaboration with Dr H. A. Krebs, to whom my best thanks are also due for instruction in the application of the manometric technique to this problem.

I am indebted to the Medical Research Council for a personal grant.

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LXXVIII. THE DETERMINATION OF DIFFUSION CONSTANTS OF PROTEINS BY A REFRACTOMETRIC METHOD.

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IN the calculation of the molecular weights of proteins from sedimentation data it is essential that their diffusion constants should be known accurately. At first diffusion constants were measured from the spreading at the sedimenting boundary in the ultracentrifuge [Svedberg, 1925, 1, 2] and the concentration changes were determined from measurements of the light absorption of the solutions. But in the ultracentrifuge it is impossible to control the temperature to a degree necessary for accurate diffusion measurements. A further objection to this method of measuring diffusion constants is that the time of diffusion in the centrifuge cell is far too short for an extended series of measurements. Therefore Tiselius and Gross [1934] applied the light absorption method to the measurement of diffusion at a boundary in a tube at rest. Small fluctuations in the results persisted due to variation of the photographic blackening caused by irregularities in the photographic emulsion or by traces of impurities in the solutions.

In the work to be described in this paper the diffusion apparatus was similar to that used by Tiselius and Gross but the concentration gradients were measured by a refractometric method developed by one of us for use in the ultracentrifuge [Lamm, 1928; 1929].

In this method a uniform transparent scale is photographed through the diffusion cell. The refractive index gradient at the diffusion boundary produces a distorted image of the scale in which scale line displacement is proportional to the concentration gradient when the refractive index is a linear function of the concentration.

The arrangement of the apparatus is shown in Fig. 1.



Fig. 1. Diffusion apparatus. *C* represents the scale and *D* the diffusion tube.

A is a quartz mercury arc lamp, *B* a filter which transmits light of a wavelength not absorbed by the solution examined, *C* the scale, *D* the diffusion cell in a water thermostat *E*, and *F* the camera. The camera objective has a focal length of 100 cm. and is placed in such a position that the scale image has a magnification of about one.

The thermostat heating element was a chrome nickel wire 70 cm. long and 0.03 cm. in diameter. It was heated by a 60-cycle current of 1.5 amperes. The water in the thermostat was well stirred and its temperature was kept constant by a Vertex contact thermometer to within 0.005°.

Details of the diffusion tube [Svedberg, 1925, 1, 2] are shown in Fig. 2. The protein solution was placed in the limb *d* and the buffer or solvent in limb *a*. The limb *a* has an inner diameter of 1 cm. The tube was placed in the thermostat and, when thermal equilibrium has been reached, the stopcock *c* was opened and the protein was forced through the capillary tube *b* by slowly increasing the air pressure on the solution in limb *d*. When the boundary between the two solutions had reached a suitable position in limb *a* the stopcock was closed. Photographs of the diffusion process were taken at suitable intervals.

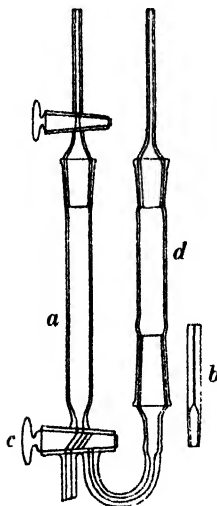


Fig. 2.

Fig. 2. Diffusion tube. The capillary *b* fits into the tube *d*. The diffusion process takes place in the limb *a*.

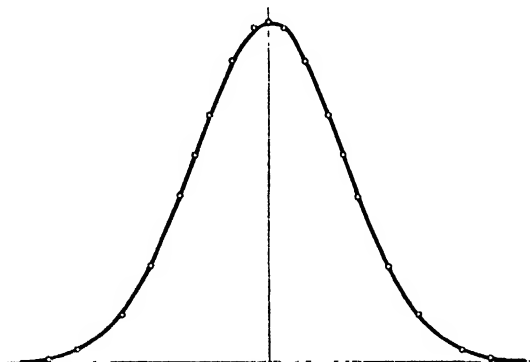


Fig. 3.

Fig. 3. Diffusion gradient curve for ovalbumin. The circles represent the ideal distribution. The abscissa axis is the direction of the diffusion process.

Method of calculation.

The scale displacements obtained from the micro-comparator readings of the distorted scale and a standard reference scale were plotted against the comparator readings of the distorted scale lines (Fig. 3)—*i.e.* the rate of change of refractive index with height was plotted against the height.

The equation for this curve has been developed by Wiener [1893] and has the form:

$$\frac{dn}{dx} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \cdot e^{-x^2/4Dt} \quad \dots\dots(1),$$

where n_1 is the refractive index of the solution,

n_0 is the refractive index of the solvent,

D is the diffusion constant,

t is the time since diffusion started,

x is the distance of a point in the cell from the original boundary.

Several equations were derived from the above equation from which the diffusion constants were calculated. We give a few equations employed:

$$D = \frac{\mu^2}{2t} \cdot \left(\frac{l-b}{l} \right)^2 \quad \text{.....(2).}$$

μ equals half the distance between the two inflection points. The position of the inflection points was obtained by dividing the maximum height of the curve by \sqrt{e} .

l represents the optical distance from the scale to the camera objective. b is the optical distance from the scale to the centre of the tube and is frequently called the scale distance. The other symbols have their previous significance.

$$D = \frac{A^2}{4\pi t H_{\max}^2} \left(\frac{l-b}{l} \right)^2 \quad \text{.....(3),}$$

where A is the area enclosed by the curve and H_{\max} the maximum ordinate of the curve.

$$D = \frac{x_1^2 - x_2^2}{4t \ln \frac{H_2}{H_1}} \left(\frac{l-b}{l} \right)^2 \quad \text{.....(4),}$$

x_1 is the abscissa at ordinate H_1 ,

x_2 is the abscissa at ordinate H_2 .

By taking H_2 as the maximum ordinate of the curve, x_2 disappears and equation (4) reduces to

$$D = \frac{x_1^2}{4t \ln \frac{H_{\max}}{H_1}} \left(\frac{l-b}{l} \right)^2 \quad \text{.....(4a).}$$

The photographic displacement (Z) of any line from its position on the undeviated scale [Lamm, 1928; 1929] is given by

$$Z = Gab \frac{dn}{dx} \quad \text{.....(5),}$$

where G is the photographic enlargement factor,

a is the inner diameter of the diffusion tube,

b is the optical distance from the scale to the centre of the tube,

Z is the photographic line displacement,

$\frac{dn}{dx}$ is the gradient of refractive index at the displaced line position.

By integration of the equation we get the relation:

$$A = \int_{-\infty}^{+\infty} \left(Gab \frac{dn}{dx} \right) dx = Gab (n_1 - n_0) \quad \text{.....(6).}$$

All the symbols have their previous significance. From this equation the refractive index increment $\frac{dn}{dc}$ of the protein solution may be calculated.

In addition to the above methods, calculation of diffusion constants was accomplished by a method which is used for obtaining the dispersion in statistical work. It depends on the assumption of treating the curves obtained from diffusion as ideal dispersions. By plotting the normal dispersion curve on that experimentally found conclusions can be drawn about the uniformity of the dispersion. The method of drawing the normal curve is that used by Pearson [1894].

The method is shortly illustrated as follows:

The base line of the curve (Fig. 3) is divided into equal units, for convenience the unit of 1 mm. was taken. We thus divide the base line into 20 to 30 units. Let the ordinates at 1, 2, 3, 4, 5, ... units be $y_1, y_2, y_3, y_4, y_5, \dots$. Then the n th

moment of the area about the vertical through the point of zero deviation is given by

$$1^n y_1 + 2^n y_2 + 3^n y_3 + 4^n y_4 \dots (7).$$

Now

$$\mu_n' = \frac{1^n y_1 + 2^n y_2 + 3^n y_3 + 4^n y_4}{A} \dots (8),$$

where A equals the area of the probability curve as before. We thus calculate μ_1' and μ_2' .

The distance of the centroid from the point of zero deviation is the ratio of its first moment to its area A and equals μ_1' . Having found the position of the centroid of the curve we calculate the second moment of the probability curve about the centroidal vertical. From μ_1' and μ_2' we calculate the standard deviation. This is given by the following relation¹.

$$\sigma = \sqrt{\mu_2} = \sqrt{\mu_2' - \mu_1'^2} \dots (9).$$

In terms of the diffusion constant the standard deviation σ equals $\sqrt{2Dt}$.

From tables compiled by Pearson [1894] the normal distribution curve can be plotted and the deviation of the experimental curve from the normal curve studied.

Preparation of the proteins.

Ovalbumin was prepared from hen's eggs according to Sørensen and Høyrup [1918], human CO-haemoglobin according to Ettish and Grosecurth [1933]. Horse serum albumin and globulin were prepared by McFarlane according to the method described by him [1935].

Erythrocrucorin was obtained from the blood of *Plumorbis corneus*.

Lactoglobulin was supplied by Dr K. O. Pedersen. The sample was prepared by Palmer [1934].

Gliadin was prepared and fractionated according to the method of Haugaard and Johnson [1931].

Before diffusion the respective protein solutions were dialysed against the buffers in order to make the electrolyte concentrations in both buffer and solution the same. Enough electrolyte was added to depress all charge effects [Tiselius, 1930].

Measurements.

Table I A gives the values obtained from an experiment with an electrolyte-free solution of ovalbumin.

In column I is given the time since the diffusion was started, column II the area enclosed by the diffusion curve and the base line, column III the maximum height of the curve, column V the diffusion constant calculated from formula (3) and column VI the diffusion constant calculated from formula (2). The symbols have their previous significance.

Table I B-H shows the results of experiments using different ovalbumin concentrations at p_H 4.6. For a buffer was used 0.02 M sodium acetate, 0.02 M acetic acid and 0.2 M NaCl.

The diffusion constants were in all cases corrected to the basis of diffusion in pure water at 20° by means of the relation

$$D \text{ corr.} = D \cdot \eta_s / \eta_w \dots (10),$$

where η_s is the viscosity of the solvent at 20°,

η_w is the viscosity of the distilled water at 20°.

¹ Sheppard [1897-98] calculated the correction which should be considered in calculating the moments of frequency curves; μ_2 in equation (9) becomes $\mu_2 - \frac{1}{12}$ when applying his correction, which is small in our present case.

Table II gives the results of an analysis of the curve obtained from the exposure after 24 hours in Table I E.

The curve was analysed by means of formula (4).

Table I. *Diffusion of ovalbumin.*

A, electrolyte-free; B-H, buffered. Bracketed average figures represent D_μ corrected according to formula (10).

Conc. of pro- tein %	Time in sec.	Area cm. ²	Max. height	$(1 - b/l)^2$	$D_A \times 10^7$ cm. ² /sec.	$D_\mu \times 10^7$ cm. ² /sec.
A. 0.88	36 000	--	--	0.871		8.06
	50 400	--	--	0.871		8.53
	86 400	183.6	15.8	0.871	7.71	8.10
	118 800	189.1	12.9	0.871	8.27	7.64
	172 800	182.7	10.35	0.871	8.00	7.72
					Average	8.01
B. 1.4	86 400	195.4	16.3	0.911	7.67	7.50
	115 200	195.2	14.3	0.911	7.50	7.50
	129 600	194.0	13.4	0.911	7.55	7.34
					Average	7.44 (7.64)
C. 0.91	21 600	189.8	29.8	0.871	8.30	7.58
	29 700	186.1	25.9	0.871	7.49	7.41
	43 200	187.2	21.6	0.871	7.89	7.50
					Average	7.50 (7.71)
D. 0.88	28 800	182.9	25.2	0.871	7.06	7.72
	43 200	180.0	20.8	0.871	7.58	7.35
	86 400	188.7	14.95	0.871	8.17	7.66
	115 200	180.0	12.9	0.871	7.50	7.46
	172 800	182.5	10.4	0.871	7.46	7.55
					Average	7.55 (7.76)
E. 0.83	79 200	170.0	14.35	0.871	7.75	7.61
	86 400	170.0	14.0	0.871	7.43	7.43
					Average	7.52 (7.73)
F. 0.7	28 800	168.2	23.3	0.871	7.66	7.57
	86 400	162.9	13.6	0.871	7.28	7.42
	115 200	161.6	11.7	0.871	7.30	7.52
					Average	7.50 (7.71)
G. 0.5	36 000	104.4	12.3	0.871	9.54	8.42
	71 100	105.2	9.2	0.871	8.73	8.16
	110 000	100.0	7.2	0.871	9.04	8.03
					Average	8.20 (8.40)
H. 0.15	14 400	48.0	7.8	0.8035	10.75	9.87
	43 200	52.0	5.0	0.8035	10.20	10.30
	86 400	52.9	3.7	0.8035	9.34	9.01
	93 600	52.6	3.6	0.8035	9.34	9.64
					Average	9.71 (9.98)

Table II.

D_μ corrected according to formula (10) = 7.75×10^{-7} cm.²/sec.

Time in sec.	H_1	H_{\max} cm.	x cm.	$(1 - b/l)^2$	$D_\mu \times 10^{-7}$ cm. ² /sec.
86 400	1.0	14	0.892	0.871	7.60
	4.0	14	0.615	0.871	7.60
	6.0	14	0.504	0.871	7.55
	8.5	14	0.385	0.871	7.45
					Average 7.55

From the foregoing tables it can be seen that D_u is constant between the concentration limits 0.7 and 1.4 % having a value of 7.71×10^{-7} cm.²/sec. Below a concentration of 0.7 % the diffusion constant increases on dilution.¹

In Fig. 3 the normal distribution curve is compared with the diffusion curve obtained from the exposure after 24 hours (Table I E). No deviations within small experimental errors are noticeable. Thus the diffusion process follows closely the ideal distribution law.

Human carbon monoxide-haemoglobin. Table III A gives the results of diffusion constants measurement on electrolyte-free CO-haemoglobin when diffused into distilled water.

Tables III B-E and IV give the results of experiments with different concentrations of CO-haemoglobin. For buffer was used 0.0085 *M* Na₂HPO₄, 0.014 *M* KH₂PO₄ and 0.1 *M* NaCl. The p_H was 6.5.

Table III.

A, electrolyte-free; B-E, buffered. Bracketed average figures represent D_u corrected according to formula (10).

	Conc. of protein %	Time in sec.	Area cm. ²	Height cm.	$(1-b/l)^2$	$D_A \times 10^7$ cm. ² sec.	$D_u \times 10^7$ cm. ² sec.
A.	0.8	39 600	122.0	13.8	0.9137	9.48	7.56
		86 400	113.0	9.0	0.9137	8.49	8.72
		97 200	110.0	8.5	0.9137	8.01	8.28
		108 000	121.0	8.3	0.9137	9.09	9.74
		129 600	113.9	7.2	0.9137	9.79	9.51
B.	3.8	86 400	536.0	47.6	0.9137	6.84	6.84
		97 200	541.8	44.5	0.9137	7.00	6.66
		158 400	524.4	35.6	0.9137	6.40	6.66
		180 000	539.4	33.6	0.9137	6.66	6.76
		Average					6.73 (6.83)
C.	2.8	57 600	391.0	42.2	0.9137	6.94	6.86
		86 400	395.0	34.4	0.9137	7.00	6.71
		100 800	399.0	32.7	0.9137	6.43	6.89
		144 000	400.0	27.4	0.9137	6.90	6.70
		172 800	396.4	25.0	0.9137	6.77	6.71
		Average					6.77 (6.90)
D.	0.82	86 800	112.5	9.9	0.9137	7.02	6.87
		100 800	118.0	9.4	0.9137	7.30	6.74
		115 200	120.0	9.1	0.9137	7.00	6.81
		172 800	114.0	7.2	0.9137	6.73	6.66
		Average					6.77 (6.90)
E.	0.4	68 400	143.5	13.6	0.8433	6.98	7.29
		86 400	144.2	12.0	0.8433	7.16	7.25
		144 000	143.0	9.5	0.8433	6.76	7.00
		154 800	140.0	8.8	0.8433	7.00	7.25
		Average					7.19 (7.34)

¹ Very little is known about this phenomenon. If the increase of the diffusion constant on dilution can be ascribed to dissociation of the protein molecules we ought to observe a fall of the sedimentation constant on dilution. This effect has been observed in the case of human CO-haemoglobin by Dr K. O. Pedersen of this laboratory [unpubl.]. Together with the authors' results (see results on CO-haemoglobin) we must conclude that CO-haemoglobin dissociates on dilution. That ovalbumin and serum albumin dissociate on dilution cannot yet be stated since the centrifugal evidence is still lacking.

Table IV.

Concentration 0.2%. D_μ corrected according to formula (10) = 7.54×10^{-7} cm.²/sec.

Time in sec.	$D_\mu \times 10^7$ cm. ² /sec.
32 400	7.65
86 400	7.31
97 200	7.38
129 600	7.21
Average	7.39

Table V. *Diffusion of 2% CO-haemoglobin against 1% CO-haemoglobin.* D_μ corrected according to formula (10) = 6.98×10^{-7} cm.²/sec.

Time in sec.	$D_\mu \times 10^7$ cm. ² /sec.
61 200	6.90
72 000	6.87
86 400	6.88
129 600	6.78
Average	6.85

Table VI. *Analysis of curve 158 400 sec. (Table III B).* D_μ corrected according to formula (10) = 6.74×10^{-7} cm.²/sec.

Time in sec.	H_{\max}	H_1	x cm.	$(1 - b/l)^2$	$D_\mu \times 10^7$ cm. ² /sec.
158 400	17.8	2.8	0.928	0.9137	6.61
158 400	17.8	4.8	0.780	0.9137	6.69
158 400	17.8	6.8	0.672	0.9137	6.76
158 400	17.8	9.8	0.528	0.9137	6.73
158 400	17.8	11.8	0.432	0.9137	6.56
158 400	17.8	13.8	0.332	0.9137	6.31
Average					6.61

From the foregoing tables it can be seen that the diffusion constant of CO-haemoglobin remains constant between the limits 3.8 and 0.8%. Below a concentration of 0.8% an increase in the diffusion constant is observed. This effect has previously been observed by Tiselius and Gross [1934] in the case of horse CO-haemoglobin.

The diffusion constants calculated from equation (3) vary very much: those calculated from equation (2) remain fairly constant.

A strong increase in the diffusion constant is observed in electrolyte-free solution and the values do not remain constant but increase with time from 7.56×10^{-7} cm.²/sec. to 9.5×10^{-7} cm.²/sec.

The diffusion constant of CO-haemoglobin has been determined by various writers. Tiselius and Gross, using the light-absorption method of Svedberg, determined a value of 6.3×10^{-7} cm.²/sec. at 20°. Northrup and Anson [1929] using the porous disc method obtained a value of 7.75×10^{-7} cm.²/sec. at 20°.

Tiselius and Gross's method of diffusing one concentration against another was followed in one experiment. The results are given in Table V. A 2% CO-haemoglobin solution was diffused against a 1% solution. No difference within experimental error was observed between this value and those in Tables III B-D.

An analysis of curve 158 400 sec. (Table III B) by means of equation (4a) gives the results tabulated in Table VI. We observe only a slight drift in the values.

A diffusion curve was compared with the normal curve (Fig. 4) but no deviation could be seen.

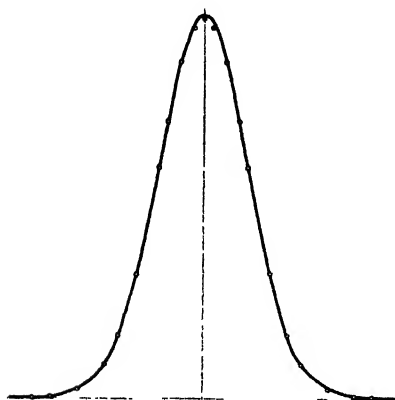


Fig. 4.

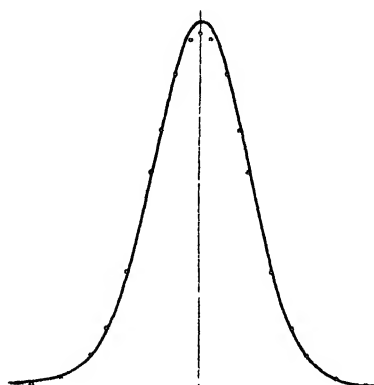


Fig. 5.

Fig. 4. Comparison between the diffusion gradient curve and the normal curve for CO-haemoglobin.

Fig. 5. Diffusion curve and ideal distribution points for serum albumin.

Serum albumin. Table VII A-C gives the results of experiments with serum albumin in different concentrations.

The buffer used was 0.019 *M* acetic acid, 0.181 *M* sodium acetate and 0.2 *M* NaCl.

In Table VIII we give the results of an analysis of curve 133 200 from Table VII B.

Table VII.

Bracketed average figures represent D_{μ} corrected according to formula (10).

	Conc. of protein %	Time in sec.	Area cm. ²	Height cm.	$(1 - b/l)^2$	$D_A \times 10^7$ cm. ² /sec.	$D_{\mu} \times 10^7$ cm. ² /sec.
A.	2.0	86 400	568.0	52.4	0.8347	5.67	5.88
		129 600	567.0	42.8	0.8347	5.77	5.80
		136 800	560.0	40.5	0.8347	5.80	5.90
		151 200	557.0	38.8	0.8347	5.80	5.89
						Average	5.87 (6.38)
B.	0.93	86 400	262.0	23.5	0.854	6.11	5.97
		133 200	264.0	19.4	0.854	5.90	5.94
		151 200	261.3	15.1	0.854	5.85	5.98
		162 000	263.0	17.7	0.854	5.80	5.92
						Average	5.95 (6.46)
C.	0.5	79 200	145.0	13.1	0.8347	6.50	6.22
		100 800	146.0	11.8	0.8347	6.48	6.23
		138 800	145.0	10.5	0.8347	5.85	5.80
		154 800	143.0	9.6	0.8347	6.11	5.95
						Average	6.05 (6.57)

Table VIII.

 D_{μ} corrected according to formula (10) - 6.46×10^{-7} cm.²/sec.

Time in sec.	H_1 cm.	H_{\max}	x cm.	$(1-b/b)^2$	$D_{\mu} \times 10^7$ cm. ² /sec.
133 200	2	19.4	0.928	0.8347	6.07
133 200	4	19.4	0.772	0.8347	6.04
133 200	7	19.4	0.612	0.8347	5.90
133 200	10	19.4	0.492	0.8347	5.86
133 200	13	19.4	0.382	0.8347	5.85
133 200	16	19.4	0.368	0.8347	5.99
Average					5.95

We observe that the diffusion constant does not vary very much between 2 and 0.93 %. At 0.5 % there is a considerable increase in the diffusion constant. The experimental curve from exposure 133 200 (Table VII B) shows a marked departure from the normal curve, see Fig. 5. It has been found that the diffusion of serum albumin followed the ideal distribution law very closely when material prepared by a different method was employed. The investigation is not completed and the results will be published in a later paper.

Serum globulin. This protein proved to be not uniform; it was composed of a main component of sedimentation constant 7.1×10^{-13} cm.²/sec. and two other components of higher sedimentation constants together with small molecules.

The protein was diffused into a buffer composed of 0.019 *M* acetic acid, 0.181 *M* sodium acetate and 0.2 *M* NaCl. p_H 5.6.

No reliable diffusion constants could be obtained as will be seen from Table IX. In Table IX a curve obtained was analysed by means of formula (4a).

Table IX.

Time in sec.	H_1 cm.	H_{\max}	x cm.	$(1-b/b)^2$	$D_{\mu} \times 10^7$ cm. ² sec
108 000	3	31	0.652	0.8543	3.61
108 000	4	31	0.588	0.8543	3.75
108 000	5	31	0.532	0.8543	3.76
108 000	10	31	0.460	0.8543	3.70
108 000	16	31	0.348	0.8543	3.62
108 000	22	31	0.240	0.8543	3.02
108 000	25	31	0.192	0.8543	2.95

From the above figures it is evident that no diffusion constant can be given. The large drift in the apparent diffusion constant is caused by the different mobilities of the differently sized particles.

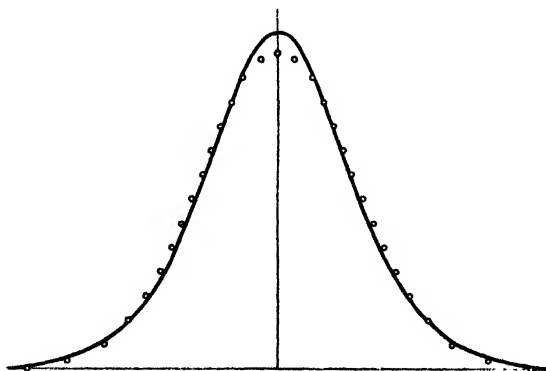


Fig. 6. Diffusion curve and ideal distribution points for serum globulin.

The diffusion curve from which the above values were obtained was compared with the normal distribution curve and a very large departure was obtained, see Fig. 6. Such a departure is characteristic for a non-uniform dispersion.

Gliadin. Gliadin was chosen to illustrate the method employed by the writers to use the method of diffusion as a test for the uniformity of a protein.

The crude gliadin obtained by extracting gluten with dilute alcohol (60% by volume) was diffused into the same concentration of alcohol.

0.1 *M* NaCl was added to depress the charge effects. The diffusion constant calculated from the standard deviation (here called the standard deviation diffusion constant) of the frequency curve gave a value of 6.04×10^{-7} when corrected for the viscosity of the solution. The diffusion curve compared with the normal curve is given in Fig. 7. There is a very large departure from the normal curve.

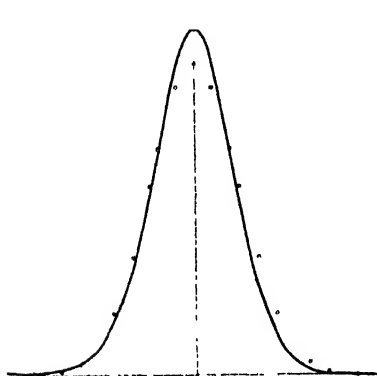


Fig. 7. Diffusion curve and ideal distribution points for crude gliadin.

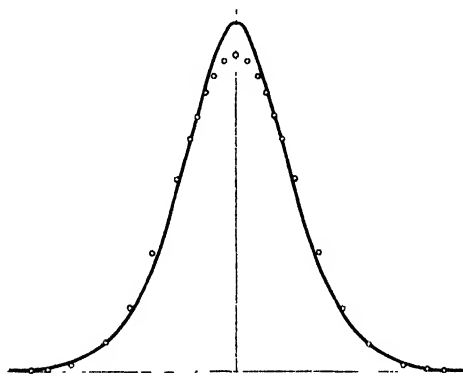


Fig. 8. Diffusion curve and ideal distribution points for 0° fraction of gliadin.

The crude gliadin solution in alcohol was cooled to 0°, and kept at that temperature for 4 days. A precipitate occurred which was purified by means of Haugaard and Johnson's method. This fraction in a concentration of 0.616% gave a normal diffusion constant of 4.96×10^{-7} cm.²/sec. This fraction proved to be very non-uniform according to the comparison of the diffusion curve with the normal curve (Fig. 8).

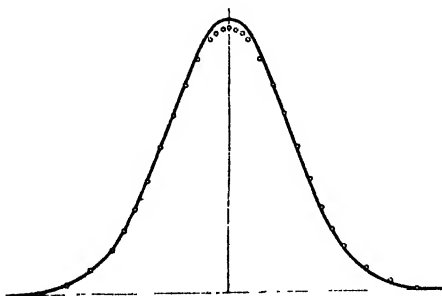


Fig. 9. Diffusion curve and ideal distribution points for -10° fraction of gliadin.

The solution obtained after the extraction of the 0° fraction was cooled to -10° and kept at this temperature for 7 days. Another fraction of gliadin

precipitated here. A 0.62% solution of this fraction after purification gave a normal diffusion constant of 5.85×10^{-7} cm.²/sec. at 20°. A diffusion curve compared with the normal curve still shows a departure (Fig. 9).

The clear solution remaining after the extraction of the 0° and -10° fractions ought to be the most uniform according to Krejci and Svedberg's [1935] ultracentrifugal study of gliadin. This fraction after purification was diffused and gave a normal diffusion constant of 6.72×10^{-7} cm.²/sec. The diffusion curve fits the normal distribution curve very well. No departure from the normal curve could be seen (Fig. 10).

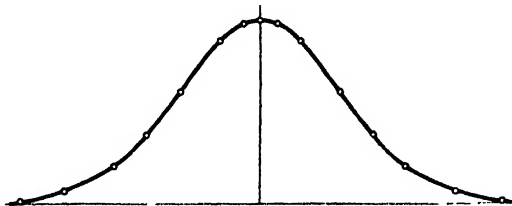


Fig. 10. Diffusion curve and ideal distribution points for the remaining fraction of gliadin. The ideal form of the curve shows the uniform character of this fraction.

According to a suggestion made by Krejci and Svedberg the gliadin remaining in solution after the extraction of the -10° fraction consists of molecules of molecular weights 34,500 and 17,000 the two molecules having the same sedimentation constant. A departure of the experimental curve from the normal curve ought to be seen if this were the case. No such evidence could be obtained from the diffusion curves.

Erythrocrucorin. The erythrocrucorin was diffused into a solvent containing 0.05 M Na₂HPO₄, 0.05 M KH₂PO₄ and 0.1 M NaCl. The p_{H} of the solvent was 6.67.

Table X gives the results on diffusing a 0.44% solution in the above-mentioned solvent.

Table X.

D_{μ} corrected according to formula (10) - 1.962×10^7 cm.²/sec.

Time in hours	$D_{\mu} \times 10^{-7}$ cm. ² /sec.
48	1.865
72	1.885
96	1.875
144	1.850
Average	1.869

D_A is omitted in favour of D_{μ} which remained much more constant as can be seen in all the previous cases.

Lactoglobulin. The lactoglobulin was diffused into solvents of different hydrogen ion concentrations. The following solutions were used:

p_H 5.0: 0.012 M acetic acid, 0.038 M sodium acetate and 0.2 M NaCl.

p_H 5.4: 0.001 M Na₂HPO₄, 0.019 M KH₂PO₄ and 0.2 M NaCl.

p_H 9.5: 0.02 M Na₂CO₃, 0.02 M Na₂B₄O₇ and 0.2 M NaCl.

A diffusion curve was compared with the normal curve. No departure was obtained.

Table XI.

Bracketed average figures represent D_μ corrected according to formula (10).

	Conc. of protein (%)	η_{sp}	Time in hours	$D_\mu \times 10^7$ cm. ² /sec.
A.	1	5.0	11.25	6.97
			17.25	6.95
			35.00	6.95
			Average	6.953 (7.18)
B.	1	5.4	17.00	6.91
			24.00	6.86
			30.00	7.00
			Average	6.925 (7.10)
C.	1	9.5	20.00	6.46
			27.00	6.50
			Average	6.48 (6.78)

DISCUSSION.

Calculation of refractive index increment $\frac{dn}{dc}$.

According to equation (6) we ought to be able to calculate the refractive index increments $\frac{dn}{dc}$ of the different proteins from the areas included by the curves with the base lines. This is verified in Table XII.

Table XII.

Ovalbumin, $\lambda = 436$ m μ .

a cm.	b cm.	U	Conc. %	Area cm. ²	Area conc.	$dn/dc \times 10^5$
1	10.1	0.865	0.50	103.2	206.4	189.0
1	10.1	0.865	0.83	170.0	204.8	187.5
1	10.1	0.865	0.88	182.5	207.3	189.8
1	10.1	0.865	0.91	187.7	206.2	188.8

CO-haemoglobin, $\lambda = 656$ m μ .

					Average	188.8
1	7	0.825	3.80	535.4	141.0	195.4
1	7	0.825	2.80	396.4	141.5	196.0
1	7	0.825	0.82	116.0	141.1	195.5
					Average	195.6

Mr K. Andersson of this laboratory kindly placed some of his determinations of $\frac{dn}{dc}$ by the Pulfrich refractometer at our disposal; for CO-haemoglobin he obtained 194×10^{-5} for the line $\lambda = 656$, and for ovalbumin for the line $\lambda = 436$ he obtained a value of 187.8×10^{-5} .

Calculation of the molecular weights.

Svedberg [1925, 1, 2; 1927] developed a formula making use of the relation between sedimentation and diffusion constants from which the molecular weights can be calculated. The relationship is the following:

$$M = \frac{RTs}{D(1 - V\rho)},$$

where M is the molecular weight,

s the sedimentation constant,

D the diffusion constant,

V the partial specific volume of the protein,

ρ the density of the solvent,

R the gas constant, and

T the absolute temperature.

Table XIII gives the molecular weights calculated from the above formula. For the diffusion constants were taken those calculated from the standard deviation. These diffusion constants will be the most reliable.

Table XIII.

Protein	$s \times 10^{13}$ cm. ² /sec.	V	$D \times 10^7$	M	
Ovalbumin	3.55	0.749	7.76	43,800	40,500*
CO-haemoglobin	4.50	0.749	6.90	63,000	68,000*
Serum albumin	4.50	0.748	6.45	67,100	66,900†
Gliadin	2.10	0.722	6.72	27,500	26,750‡
Erythrocyruorin	33.70	0.745	1.96	1,634,000	1,539,000§
Lactoglobulin p_H 5.0	2.95	0.751	7.18	40,000	37,800*
Lactoglobulin p_H 9.5	2.76	0.751	6.78	39,600	---

* [Pedersen, unpubl.].

‡ [Kreji and Svedberg, 1935].

† [Mutzenbecher, 1933].

§ [Eriksson-Quensel, 1934].

In the last column of the above table the molecular weights as determined by Svedberg's equilibrium centrifuge method are given. We draw attention to the fact that the sedimentation and diffusion constants of lactoglobulin both fall at p_H 9.5 giving the same molecular weight as that found at p_H 5.0. The particle most probably changed its shape thereby acquiring a higher frictional constant.

SUMMARY.

1. A refractometric method has been described for measuring diffusion constants of proteins.

2. Several methods for calculating the diffusion constants from the curves are discussed.

3. The diffusion constants of several well-known proteins have been determined, *viz.* ovalbumin, human CO-haemoglobin, serum albumin, gliadin, erythrocyruorin (*Planorbis cornutus*) and lactoglobulin. It has been found that the diffusion constants of ovalbumin, CO-haemoglobin and serum albumin increase very much in dilute solutions below 0.5 %.

4. A method used in statistical work for obtaining the dispersion has been applied to the diffusion curves. It has been found that in monodisperse solutions the diffusion follows the ideal dispersion law very closely. In cases where we diffused polydisperse solutions we obtained departures from the normal curves.

5. From the areas included by the diffusion curves we have calculated the increments of refractive indices. The values obtained agree very closely with those determined by K. Andersson.

6. From the diffusion and sedimentation constants the molecular weights have been calculated, the values obtained agree well with those found by the equilibrium centrifuge.

To the chief of the laboratory, Prof. The. Svedberg, the authors express their sincere thanks for his unfailing interest and for the facilities provided for this work in his laboratory.

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LXXIX. LIVER GLYCOGENASE.

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(Received January 30th, 1936.)

THE mechanism of the utilisation of liver glycogen has not been so extensively worked out as that of muscle glycogen. The purpose of the present work is to study the action of liver glycogenase by making an active preparation of the enzyme, determining the end-products of its action on glycogen and observing some of the factors influencing this reaction.

Various means of preparing glycogenase have been used. The extracts made by the Buchner (hydraulic press) method are very unstable because they contain proteolytic enzymes which quickly destroy the glycogenase, even when the extracts are kept at a low temperature. Another method of preparation is to grind some of the tissue thoroughly in a mortar, using quartz sand, and then to mix with several volumes of alcohol. The resulting precipitate after standing under alcohol for a few days is collected on a filter-paper and dried *in vacuo*. A weighed portion of the powder can then be taken at any time and ground up into a homogeneous suspension with water. The chief objection to this method is that contact with alcohol appears to alter the potency of the precipitate so that if it is to be used for purposes of comparison care must be taken to see that all the conditions are exactly the same.

In the method of Wicchowski [1910] the tissue is very rapidly macerated to a fine pulp which is spread out on glass plates and these are placed in a rapid current of warmed air until completely dry; the crust is then removed and the scales thoroughly extracted in a suitable extractor with toluene.

More recently Eadie [1927] made an enzyme preparation in which the liver was thoroughly ground, then treated twice with acetone, once with a mixture of equal parts of acetone and ether and finally twice with ether. After drying overnight the powder thus obtained was extracted for several days with 50% glycerol, filtered and dialysed against running water for about 5 hours. The resultant solution was frequently found to be unstable, but it could always be used for 24 hours. When 5 ml. of enzyme solution were allowed to act for 5.25 hours on 5 ml. of 1.5% glycogen solution, the reducing sugar calculated as glucose was about 10% of the amount for total hydrolysis. This amount of reducing sugar represents the hydrolysis of only a small part of the glycogen and indicates that the enzyme preparation is a comparatively inactive one.

The action of glycogenase has been measured by determining from time to time either the amount of glycogen left unchanged or the amount of sugar formed. The former method being somewhat laborious, Salkowski [1906] and Wohlgemuth [1908] elaborated methods in which starch, not glycogen, was used as the substrate, and the hydrolysis was followed quantitatively by the starch-blue reaction with iodine. This procedure is open to criticism because it is based on the unwarranted assumption that starch has the same properties as glycogen (see Haworth [1929]).

Experiments in which the action of pancreatic amylase on glycogen has been followed are also open to criticism, because they are based on the assumption that glycogenase acts exactly like amylase.

METHODS.

The enzyme was prepared from the liver of rabbits. The rabbits were killed by severing the carotid artery on one side with a sharp razor and allowing them to bleed. The liver was removed, cut into fine pieces and pressed through cheese cloth in a mortar with twice its weight of acetone. The acetone extraction was repeated once. The acetone extract was filtered, and the residue, which rapidly dried, was put through a sieve. The fine powder thus obtained is a potent preparation of the enzyme.

To study the properties of the enzyme a weighed quantity of the powder was added to a buffered (Clark and Lub) solution of glycogen. The glycogen was prepared from rabbit's liver by the method of Sahyun-Alsberg [1930] and dialysed against running water. The flask containing the mixture was placed in a water-bath at 37° and shaken mechanically. Reducing sugar in samples from the mixture was determined by the Shaffer-Hartmann [1921] method. Glycogen was determined by heating samples from the mixture with an equal quantity of 60% KOH in a boiling water-bath for 3 hours, precipitating by alcohol, hydrolysing with 2.2% HCl, and estimating the glucose by the Shaffer-Hartmann method. Thus where simultaneous estimations of substrate and end-product were undertaken the same method for estimating glucose, the Shaffer-Hartmann, was used and possible errors thereby diminished.

EXPERIMENTAL.

A preliminary experiment was carried out to determine the relative amounts of enzyme in the liver of fasted and of fed animals. Accordingly four animals from the same litter were selected and two fed and the others fasted for 2 days; all four were then killed and enzyme preparations made from their livers. That from the fed animals contained a certain amount of glycogen. That the preparations from the fasted animals contained no glycogen was demonstrated by adding some to Clark and Lub buffer solutions and finding no reducing sugar in the mixture over a 5-hour period.

Into each of four flasks were placed 30 ml. of Clark and Lub buffer p_H 7.5 and 1.0 g. of enzyme preparation from each of the four livers. To each flask containing enzyme from the fasted animals was added 0.45 g. of glycogen. The flasks were shaken in a bath at 37° and samples taken and analysed for glycogen at 0, 0.5, 1.5, 3 and 5 hours (see Table I).

Table I.

Time (hours)	% glycogen (as glucose)			
	Fed rabbit	Fed rabbit	Fasted rabbit	Fasted rabbit
0	1.31	1.35	1.46	1.43
0.5	0.93	0.87	0.71	0.73
1.5	0.56	0.53	0.26	0.30
3	0.30	0.29	0.12	0.13
5	0.16	0.12	0.16	0.06

The reaction proceeded faster with the preparations from the fasted animals than with those from the fed animals, but the amount of enzyme in the extracts from the former was greater than that in the latter, and when this allowance is made it will be seen that deprivation of food does not appreciably affect the enzyme content. This is an observation of importance because it renders possible

the preparation of glycogenase free from its substrate glycogen. Hence the enzyme preparations used in the succeeding experiments were derived from the livers of fasted animals.

Influence of p_H .

To each of three flasks containing 30 ml. of Clark and Lub buffer of p_H 6.0, 7.0 and 8.0 with glycogen in solution was added 1.0 g. of enzyme preparation. These were shaken in a bath at 37° and samples taken both for glycogen and sugar at 0, 0.5, 1.5, 3 and 5 hours.

The results are recorded graphically in Fig. 1. This shows that the reaction proceeds more quickly in an alkaline medium.

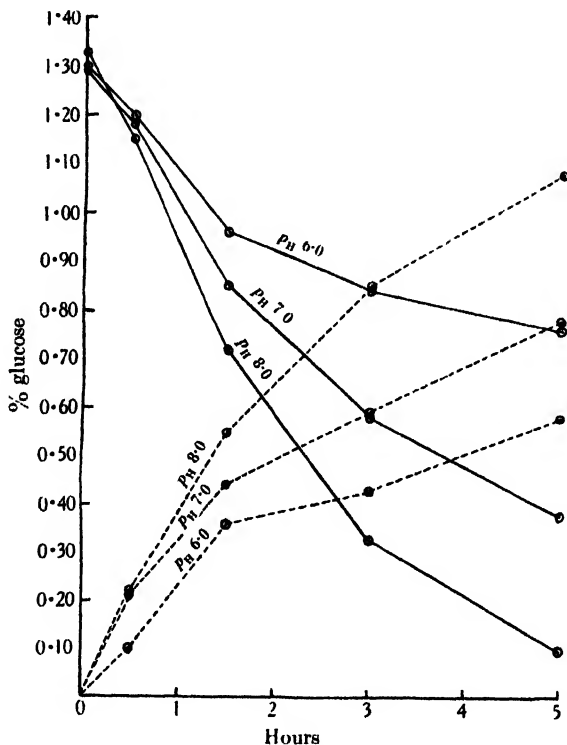


Fig. 1. The relation of glycogenolysis to sugar formation.

— — Glycogen (calculated as glucose). - - - Reducing sugar (calculated as glucose).

End-product of the reaction.

A further study of the graph disclosed the fact that at any time the contents of the mixture may be expressed in two parts, material estimated as glycogen and reducing sugar, and that the sum of the two parts is approximately equal to the amount of glycogen (expressed as glucose) to start with. This would not be true were maltose present. If the reducing power of glucose with copper reagents be represented by 100, that for maltose may be taken as 55. Hence the presence of maltose would cause a much lower percentage of reducing sugar to be shown at any one time, and the two fractions would not be complementary. Therefore, either no maltose is formed, or, if formed, the rate of its destruction equals that of its formation.

Influence of blood on the reaction.

Eadie [1927] questioned the results of previous workers on the action of liver glycogenase on the grounds that their enzyme preparations contain blood amylase. He performed experiments using a rat liver which had been perfused for 1 hour with warmed Ringer solution and observed an optimum p_H for the enzyme at a lower level than that found by other workers. However, it is known that washing out the liver may either reduce the activity of the enzyme, or destroy it in part, and Davenport [1926] pointed out the fallacy of comparing the activity of unmodified blood serum with blood which has been through an extraction process. In order to investigate this point a quantity of rabbit's blood was subjected to exactly the same procedure as the liver. When the powder thus obtained was allowed to act on a buffered solution of glycogen no reducing sugar was formed over a 5-hour period. Thus the method of extraction which is here employed yields a potent liver glycogenase but destroys blood amylase, and there can be no interference in these experiments by the latter enzyme.

In another experiment 5 ml. of rabbit's blood serum were allowed to act for 5 hours on a 0.2%, buffered solution of maltose; over this period there was no change in the reducing power of the maltose solution, thus indicating no maltase effect.

Effect of the enzyme on maltose.

The preparation of the enzyme free from glycogen makes it possible to obtain a significant result from its action on maltose and lactose. Accordingly to each of four flasks, of which three contained 30 ml. of 0.2% maltose in Clark and Lub buffered solutions, p_H 6.0, 7.0 and 8.0 respectively, and one contained 30 ml. of 0.2% lactose solution in Clark and Lub buffer solution p_H 7.0 was added 1.0 g. of enzyme preparation.

The tubes were shaken in a bath at 37° and samples analysed for reducing sugar at 0, 0.5, 1.5, 3 and 5 hours (see Table II).

Table II.

Time (hours)	% reducing sugar (as glucose)			
	Maltose mixtures			Lactose mixture
	p_H 6.0	p_H 7.0	p_H 8.0	p_H 7.0
0	0.110	0.113	0.112	0.144
0.5	0.160	0.147	0.151	0.146
1.5	0.188	0.178	0.184	0.148
3	0.201	0.195	0.197	0.148
5	0.203	0.199	0.201	0.147

The enzyme thus appears to be almost equally active on maltose over a range p_H 6.0–8.0. It is possible however that either there was an excess of enzyme present, thus masking the effect of p_H , or that the optimum p_H lies outside the range investigated.

The enzyme was without effect on lactose at p_H 7.0.

Complete hydrolysis of the mixtures was carried out at the end of the 5-hour period. To 2 ml. of mixture were added 2 ml. of 2.2% hydrochloric acid and the whole allowed to boil gently under a reflux condenser for half an hour. The solution was then neutralised with sodium bicarbonate and analysed for reducing sugar. The results of this procedure gave values of 0.206, 0.210, 0.203 and 0.206% respectively, all close to the theoretical. It will thus be seen that almost the whole of the maltose was changed to glucose by the activity of the enzyme.

Influence of sodium chloride.

Four flasks containing glycogen in solution in 30 ml. Clark and Lub buffer p_H 7.4 were prepared and sodium chloride to 0.1, 0.5 and 1.0 % added to three of them. They were shaken in a bath at 37° and samples analysed for sugar at 0, 0.5, 1.5, 3 and 5 hours (see Table III).

Table III.

Time (hours)	% glucose			
	Control	With 0.1 % NaCl	With 0.5 % NaCl	With 1.0 % NaCl
0	0	0	0	0
0.5	0.285	0.250	0.260	0.203
1.5	0.591	0.575	0.580	0.493
3	0.875	0.825	0.845	0.818
5	0.920	0.915	0.920	0.905

These results show that sodium chloride has no appreciable effect on the rate of reaction.

Effect of dialysis.

3 g. of enzyme preparation were dialysed in a collodion sac against distilled water for 48 hours with one change. Three flasks containing glycogen in 30 ml. of Clark and Lub buffered solution p_H 7.4 were taken: to the first was added 1.0 g. of undialysed enzyme preparation, to the second 1.0 g. of dialysed enzyme preparation: and to the third 1.0 g. of dialysed enzyme and sodium chloride to 0.1 %. The flasks were heated in the usual way (see Table IV).

Table IV.

Time (hours)	% glucose		
	Control mixture	Dialysed enzyme mixture	Dialysed enzyme mixture with 0.1 % NaCl
0	0	0	0
0.5	0.280	0.02	0
1.5	0.645	0.09	0.02
3	0.825	0.11	0.05
5	0.930	0.12	0.09

This experiment shows that dialysis destroys the activity of the enzyme and that its activity is not restored by the addition of sodium chloride.

Effect of insulin on glycogenase.

Cambridge and Howard [1924] showed that insulin inhibited the rate of hydrolysis of starch *in vitro* by liver amylase. Visscher [1926], using glycogen as a substrate, reported only slightly less hydrolysis in experiments in presence of insulin. Popper and Wozasek [1933] observed decreased diastase content in fatal insulin hypoglycaemia.

To investigate this problem four animals from the same litter were selected and fasted for 2 days. Two of the animals were each given 12 units of insulin subcutaneously and 2 hours later 12 units intravenously. Both of these were killed in convulsions and the two control animals killed simultaneously. Enzyme preparations were made from the livers, and 1.0 g. extract was added to each of four flasks containing 30 ml. of Clark and Lub buffer p_H 7.4 with glycogen in solution, which were treated in the usual way (see Table V).

Table V.

Time (hours)	% glucose			
	Control	Control	Insulinised animals	
0	0	0	0	0
0.5	0.254	0.230	0.200	0.195
1.5	0.650	0.607	0.460	0.483
3	0.750	0.730	0.570	0.580
5	0.780	0.760	0.635	0.640

The small decrease in the activity of the enzyme which followed the lowering of the blood sugar to the convulsive stage is of doubtful significance. It is therefore improbable that insulin, under physiological conditions, has any effect on glycogenase. This is to be expected if insulin does not influence liver glycogenolysis.

Regarding the effect of adrenaline, Langfeldt [1921] claimed that adrenaline not only increased the activity of liver amylase but also altered its optimum hydrogen ion concentration. Later workers could substantiate neither effect, and the present view is that no *in vitro* effect of adrenaline can be demonstrated.

DISCUSSION.

The results obtained show that liver glycogenase converts glycogen quantitatively into glucose, independently of any amylase action on the part of the blood, and that, unlike amylases, glycogenase is not appreciably influenced by variations in the sodium chloride content of the medium. It would also appear that as quickly as maltose is formed it is converted into glucose.

Many attempts have been made to explain how glycogenase and glycogen can exist together in the liver cell. Macleod [1926] advanced the view that glycogenolysis was set up by the local production of a certain amount of acid, and that such a liberation of free acid could be brought about by a curtailment in the arterial blood supply of the hepatic cell, vasoconstriction either from adrenaline or nervous stimulation being suggested as a probable cause. It is thus assumed that at the normal p_H of the liver cell the enzyme is inactive and that with a change in p_H it increases enormously. It has been shown in the present work that the enzyme is by no means inactive at the p_H of the blood, and that its activity is but little altered over any range of p_H likely to be encountered under physiological conditions.

Lesser [Lesser and Kerner, 1920; Lesser, 1920; 1921] advocated the view that the glycogen and amylase were "locked up" in different compartments of the cell. He showed [Lesser and Zipf, 1923] that homologous alcohols in isocapillary concentrations increased sugar formation in the perfused frog's liver by approximately the same amount; that this effect occurred under reversible conditions [Lesser, 1925]; and that the effect was also similar in a perfusion fluid buffered with CO_2 and bicarbonate, p_H 7.4-7.6 [Lesser, 1926]. On the basis of these findings he concluded that a boundary surface phenomenon was involved, and he assumed that two-thirds of the enzyme was adsorbed on the surfaces of the cell and therefore unable to act on the liver glycogen.

On the basis of this view the rôle of adrenaline in glycogenolysis may be considered to be the release of glycogenase by physico-chemical action from intracellular interfaces.

SUMMARY.

1. A method of preparation of glycogenase from rabbit's liver is described.
2. The enzyme converts glycogen into glucose, the rate of reaction being greatest on the alkaline side of neutrality.
3. Maltose is quantitatively changed into glucose by the enzyme.
4. Sodium chloride has no significant effect on the rate of reaction.
5. The enzyme is destroyed by dialysis and its activity is not restored by sodium chloride.
6. No significant decrease in the glycogenase in the liver was observed to follow administration of convulsive doses of insulin.
7. The bearing of these experiments on the problem of glycogenolysis is discussed.

My thanks are due to Prof. J. Mellanby for kind help and advice.

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LXXX. THE PROTEOLYTIC ENZYMES OF SPROUTED WHEAT.

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(Received December 9th, 1935.)

ALTHOUGH previous work has shown the presence of proteolytic activity in wheat and wheat flour, attention has been focused on the fate of the wheat proteins rather than on the nature of the enzymic processes [Sharp and Elmer, 1924; Johnson *et al.*, 1929; Brownlee and Bailey, 1930]. Although the proteolytic activity of wheat is very slight [Cairns and Bailey, 1928; Herd, 1931; Landis, 1935] it may be enhanced considerably by germination of the seeds [Bach and Oparin, 1922]. In an endeavour to compare the proteolytic enzymes of wheat with those demonstrated in barley and malted barley [Hopkins *et al.*, 1929; 1930; 1931; Linderstrom-Lang *et al.*, 1929; Sato, 1931] the principal characteristics of a protease and a dipeptidase have been determined in aqueous extracts of germinated wheat seeds. The results serve to confirm and amplify the similar conclusions of Pett [1935] which have appeared very recently.

EXPERIMENTAL.

Various methods are available for the measurement of proteolytic activity [Cairns and Bailey, 1928] and of these the determination of liberated amino- or carboxyl groups by the Sorensen formaldehyde titration was chosen, as the procedure is simple and gives accurately a set of data which has a recognised meaning. The comparator technique of Grünhut [1919] and Lüers [1920], slightly modified, was found satisfactory. Formaldehyde concentration at the final end-point was 16-20 % [Harris, 1929] and indicator concentration was carefully maintained at one drop of 0.2 % phenolphthalein per ml. solution in the comparator tube. Titration of aliquots at stated time intervals was carried out with *N*/20 NaOH in two stages: (a) directly in aqueous solution to an end-point corresponding to p_H 8.5 (compared with a buffer solution of that p_H), and (b) from this end-point to the same colour after formaldehyde addition. Titre (b) gives a first approximation to amino-N liberated by proteolysis, while titre (a) + (b) represents liberated carboxyl groups [Grünhut, 1919; Richardson, 1934; 1935]. Since titre (b) will be deficient with respect to some of the polypeptide amino-N (which is already partly titrated in the (a) titration), titre (a) + (b) is probably a more reliable estimate of the total scission of peptide linkages and has been used in the present work except where otherwise stated.

Preparation of enzyme extract. The naturally feeble proteolytic activity of wheat seeds was reinforced by germination for not longer than 5 days; with longer periods mould growth was occasionally observed on the damp wheat. Uniformity of germination was secured by the following procedure: 150 g. of English wheat (1934 crop) were soaked in an excess of tap water for 24 hours at 18°, after which the grains were transferred to perforated porcelain germinating trays and kept covered with moist absorbent cotton-wool for 4 more days.

After weighing, the germinating seeds were minced finely and the gain in weight (usually about 90%) was made up to 100% with distilled water. The mass was finally extracted for 2 hours at 30° with an additional 200 ml. distilled water containing a few drops of toluene and filtered. The clear golden-brown filtrate was stored, with additional toluene, in the dark at 18°. It invariably darkened in colour and deposited a fine dark brown precipitate, the change usually being noticeable within 24 hours. Loss of activity (Fig. 6) and changes in p_H with age necessitated the use of freshly prepared extracts only.

p_H measurement. All p_H values were determined with a glass electrode of the Hughes [1922] type coupled to a Harrison [1930] triode valve amplifier unit.

Reaction conditions. Edestin and leucylglycine were chosen as substrates for the investigation of protease and peptidase activities respectively. Reaction mixtures, except where specified, had a total volume of 35 ml. and consisted of varying quantities of edestin dissolved in $N/5$ acetic acid together with enzyme and appropriate acetic acid-sodium acetate buffers. The enzyme extract, brought to 40°, was added from a specially calibrated pipette to the mixed substrate and buffer solutions, also at 40°. Flasks were closed with cork stoppers since rubber ones were shown by Sato [1931] to retard the reaction in the case of malted barley protease. 5 ml. samples were withdrawn within 2 min. and again after 4 hours at 40° and titrated with $N/20$ NaOH as previously indicated.

Control tests. Control solutions for each reaction mixture were made up as follows: (1) substrate + buffers + boiled enzyme, (2) substrate + buffers only, (3) buffers + fresh enzyme only.

The increases in (1) and (2) were always negligible. (3) however almost invariably provided an appreciable correction titre (0.07–0.10 ml. NaOH in the protease experiments and up to 0.32 ml. in the corresponding dipeptidase controls, Fig. 8). These values are no doubt attributable to the action of the proteolytic enzymes of the wheat extract on those wheat proteins rendered soluble under the conditions of extraction (*e.g.* leucosin and globulin). Toluene was used throughout as antiseptic.

RESULTS.

A. *Protease.*

Determination of p_H optimum for edestin. The effect of variation in p_H on protease activity was determined by the formaldehyde titration method as outlined above and checked by Harris's [1923] method of titration in 85% alcohol using thymolphthalein as indicator (Fig. 1). Both curves indicate an optimum at about p_H 4.1.

Rate of reaction. The rates of activity of protease at 30° and 40° respectively are compared in Fig. 2.

Relation between initial reaction velocity and substrate concentration. The saturation point of wheat protease for edestin as substrate is shown in Fig. 3. The curve is constructed from values for increase in titre (*b*) only. Details as in Fig. 2 except for substrate concentration.

Effect of variation in enzyme concentration with constant substrate concentration. Fig. 4 indicates influence of gradually increasing enzyme concentration when substrate concentration is maintained constant, and in Table I are presented values for Schutz's constant (K) calculated from $x = K\sqrt{et}$ (where x = amount of protein digested, e = enzyme concentration, and t = time).

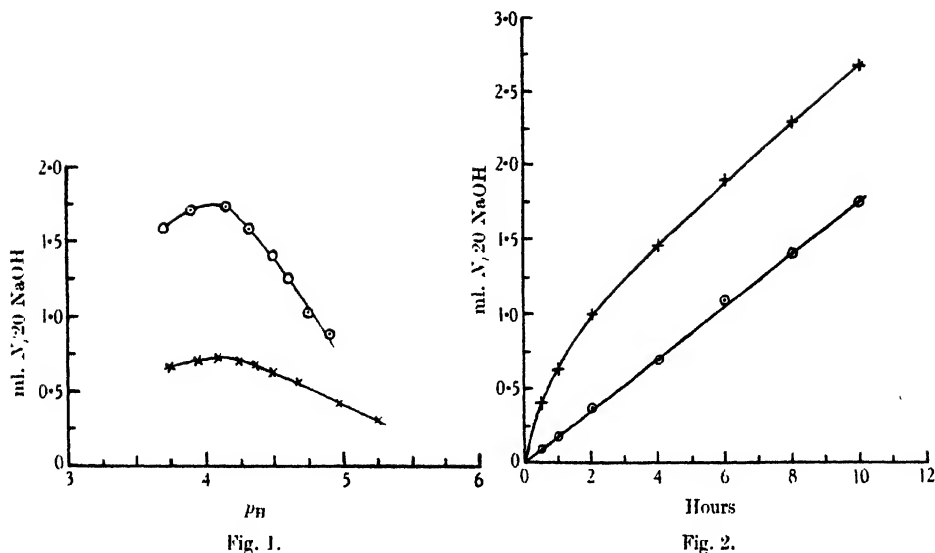


Fig. 1. Variation of protease activity with pH . Conc. of edestin in reaction mixture: 1.4%, $M/7$ acetate buffer. Enzyme dilution 2 : 7. Titres as ml. $N/20$ NaOH per 5 ml. sample in excess of a control solution without edestin. \times Formaldehyde titration of 4-hour samples. \circ Alcohol titration of 10-hour samples.

Fig. 2. Effect of temperature. Edestin 1.4%, Enzyme dilution 2 : 7. $M/7$ acetate buffer at pH 4.1. \circ — \circ 30° ; \times — \times 40° .

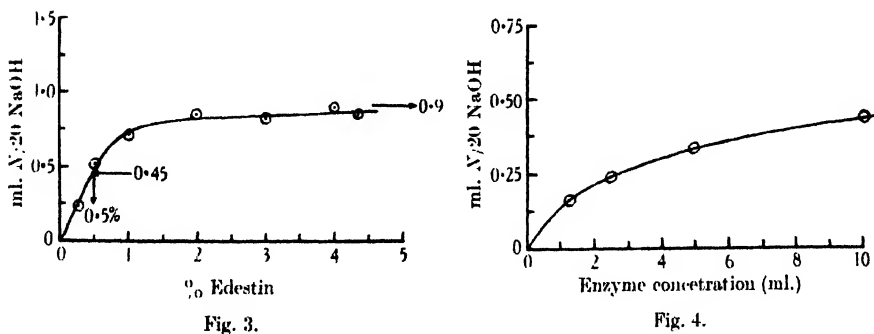


Fig. 3. Effect of edestin concentration. Formaldehyde titration of 4-hour samples.

Fig. 4. Effect of enzyme concentration. Edestin 0.7%, $M/7$ acetate buffer at pH 4.1. a ml. enzyme, $(10 - a)$ ml. water. Formaldehyde titration of 4-hour samples.

Table I.

Enzyme concentration in ml. (= e)	1.25	2.5	5.0	10.0
Titre (b) (increase ml. $N/20$ NaOH = x)	0.16	0.24	0.34	0.44
Schutz's constant (K)	0.072	0.076	0.076	0.070

Heat-inactivation of wheat protease. The critical inactivation temperature, *i.e.* the temperature at which an enzyme is half destroyed in 1 hour, was determined by heating quantities of enzyme extract to 40, 50, 55 and 60° respectively before adding to the substrate-buffer mixture. For this purpose, 12 ml. fresh extract at its natural p_H value of 6.0 were heated as rapidly as possible to the desired temperature, maintained there for 1 hour and cooled rapidly to 18°. Evaporation losses were made good with distilled water and 10 ml. of well-mixed extract transferred to reaction flask.

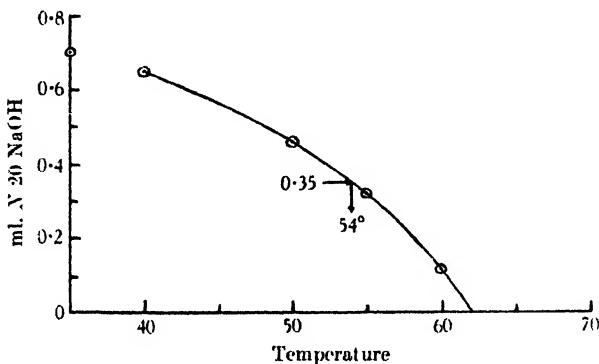


Fig. 5. Critical inactivation temperature of protease. Edestin 2.1%, Enzyme dilution 2:7. M/7 acetate buffer at p_H 4.1. Normal value, 0.70 ml. N/20 NaOH.

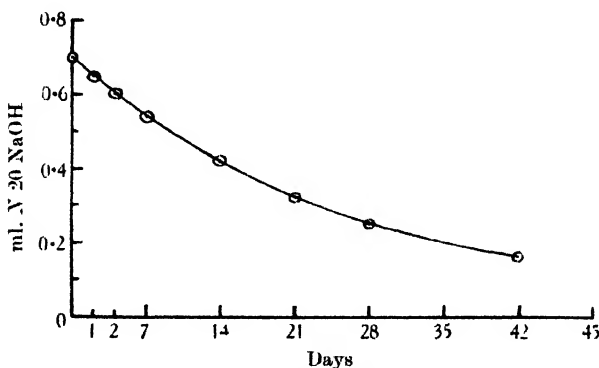


Fig. 6. Effect of keeping.

Inactivation of wheat protease with age. The storage of an aqueous extract of sprouted wheat in the dark at 18° and in the presence of toluene results in progressive loss of activity: the rate of loss is depicted in Fig. 6 (details as for Fig. 5). Increases are those of titre (*b*) only. In Table II are calculated the values for *k* (referred to later) from the equation $k = \frac{1}{t} \log \frac{a}{a-x}$.

Table II.

Days of storage ...	0	2	4	7	14	21	28	42
Increase in titre (ml. N/20 NaOH)	0.70	0.65	0.60	0.54	0.42	0.32	0.25	0.16
Velocity constant <i>k</i>	--	0.0161	0.0167	0.0161	0.0159	0.0162	0.0160	0.0153

On ageing, the extract deepened in colour and a brown precipitate appeared. Moreover, the p_H value of the extract examined fell from 6.04 to 5.17 in the 42 days occupied by the tests.

Inhibition of protease action. Attempts to use buffers such as sodium citrate-HCl, potassium hydrogen phthalate-HCl, and glycine-NaCl-HCl, in place of acetic acid-sodium acetate mixtures in the preceding experiments met with little success, increases in titre being either nil or but a small fraction of the corresponding value in the presence of acetate buffers. It will be evident that all except the latter mixtures contain HCl as common ingredient and it therefore seemed reasonable to suppose that chloride ions were responsible for the inhibitory effect. Fig. 7 shows the influence of varying concentrations of NaCl on protease activity. The graph is drawn from data for increase in titre (*b*) only. A thick precipitate of edestin was observed in those mixtures containing 0.2 and 0.3 *M* NaCl, a slight precipitate in that containing 0.1 *M* NaCl, whilst the remaining ones were clear.

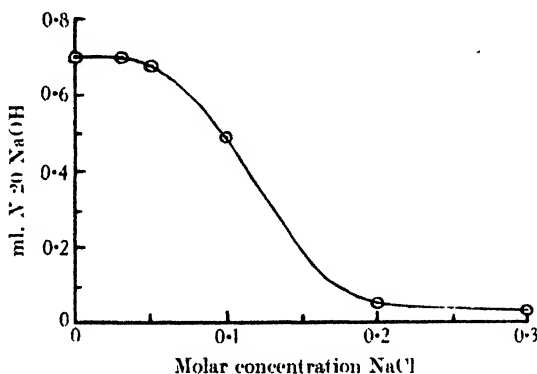


Fig. 7. *Effect of NaCl on protease activity.* Edestin 2.0%. Enzyme dilution 2:7. *M*/7 acetate buffer at p_H 4.1. \times ml. 10% NaCl. (5-5) ml. water.

It should be pointed out that the use of sodium citrate-citric acid buffers also yielded no increase in titre at p_H 4.1. Data have also been obtained which show that phosphate ions slightly reduce the activity of protease.

Investigations now in progress have established that wheat protease is markedly activated by cyanide in concentrations as low as 0.005 *M*, the p_H optimum at the same time being shifted towards the alkaline side. It is hoped to publish full confirmation later.

B. Dipeptidase.

The existence of a dipeptidase in the aqueous extract of sprouted wheat is demonstrated in the following experiments by the use of glycylglycine and leucylglycine as substrates. The formaldehyde comparator technique was again adopted for all mixtures having a p_H value below 8.5. For more alkaline values back-titration was practised: to the 5 ml. sample + indicator in the comparator tube was added a known quantity (usually 1 or 2 ml.) of *M*/20 potassium hydrogen phthalate sufficient to reduce the alkalinity to below p_H 8.5. Titration then proceeded with *N*/20 NaOH to the usual end-point.

The buffers used were acetic acid-sodium acetate, potassium dihydrogen phosphate-NaOH, dipotassium hydrogen phosphate-NaOH. In addition, simple mixtures of NaOH and water were tried, the natural buffering effect of the

enzyme extract being found sufficient to maintain remarkably constant p_H values throughout the reaction period, which in these experiments was 3 hours instead of 4.

p_H optima for leucylglycine and glycylglycine. The corrected curves in Figs. 8 and 9 indicate the respective p_H optima for leucylglycine and glycylglycine, namely 7.3 and 7.9. In each figure are included the experimental test and control curves from which the corrected curves were calculated by difference. The control curve was determined on mixtures containing no dipeptide.

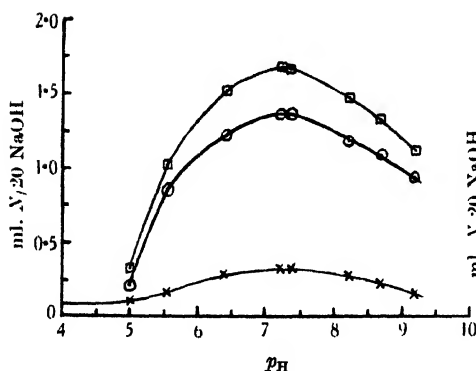


Fig. 8.

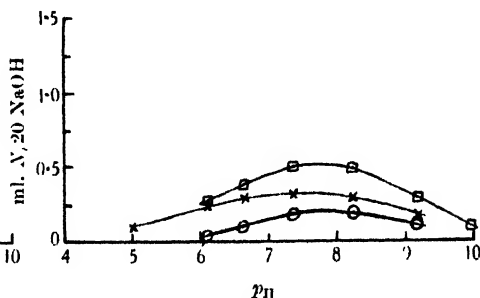


Fig. 9.

Fig. 8. p_H optimum for leucylglycine. Leucylglycine $M/25$. Enzyme dilution 2:7. $M/10$ phosphate-NaOH buffers. Titration of 3-hour samples. \square Test curve. \times Control curve. \circ Corrected curve.

Fig. 9. p_H optimum for glycylglycine. Glycylglycine $M/25$. Other details as in Fig. 8.

Stability of dipeptidase. The inactivation of dipeptidase on storage of the aqueous extract of sprouted wheat at 18° in the dark and in the presence of toluene is shown in Table III, both leucylglycine and glycylglycine being used as substrate. Details as in Fig. 8.

Table III.

Days of storage	0	1	2	3	4
Increase in titre	{	Leucylglycine	1.34	1.16	0.62	--	0.06
		Glycylglycine	0.19	0.07	0.01	0.02	--

DISCUSSION.

It is clear from the experimental data that an aqueous extract of sprouted wheat contains at least two proteolytic enzymes, a protease and a dipeptidase, of which the former functions at acid, and the latter at alkaline, reactions. This accords with the results of Linderstrøm-Lang *et al.* [1929; 1932; 1934] on aqueous extracts of malted barley, in which the existence of similar enzymes was established. The corresponding p_H optima, namely 4.1 and 4.3 for the action of wheat protease and malt protease respectively on edestin at 40° , and p_H 7.3 and 8.6 for the action of wheat dipeptidase and malt dipeptidase respectively on leucylglycine at 40° , suggest substantial similarity between the proteolytic enzyme equipment of the two cereals. Furthermore, the behaviour of the two enzymes in the aqueous wheat extract during storage resembles that of the malt extract, in that the protease loses some activity but is much more stable than the dipeptidase. The stability of an enzyme is, of course, influenced

by several factors, such as temperature of storage, condition of the preparation, extent of exposure to light, p_H value during storage, presence of stabilisers such as glycerol *etc.* Tables II and III deal only with one set of conditions, namely storage of aqueous extracts at 18° with toluene and in the dark. Experiments now in progress point to a much higher stability of the dipeptidase in the presence of glycerol. The rate of destruction of the protease is slightly more rapid in the early stages of storage, the activity being halved in about 18 days. The graph in Fig. 6 bears a distinct resemblance to an inverted unimolecular reaction curve and, assuming a = the initial rate of proteolysis and x = diminution in activity in time t , the application of $k = \frac{1}{t} \log \frac{a}{a-x}$ yields values for k which are reasonably constant (see Table II) and indicate that spontaneous inactivation of wheat protease follows a unimolecular course. This suggests simple disintegration of the enzyme complex rather than its inactivation by combination with any other substance.

The course of proteolysis is generally too complex a process to follow any simple formula, yet Schutz [1885] found that the digestion of egg albumin by pepsin, as measured by peptone formation, varied as $\sqrt{\text{enzyme concentration}}$. Arrhenius [1907] extended this rule and derived the formula $x = K\sqrt{et}$ (where x = amount of protein digested, c = enzyme concentration, t = time and K is a constant). He found this to apply over a considerable range so long as t is neither too small nor too large. The application of this formula to the present work yields values for K which are fairly constant (Table I), so that during the initial stages, at least, the action of wheat protease on edestin appears to follow the Schutz-Arrhenius rule. The formula, unfortunately, does not offer much further insight into the nature of proteolysis. Nor is much help gained from the equation $K_m = x \left(\frac{V}{v} - 1 \right)$ (where x = concentration of substrate, v = velocity of reaction, V = limiting velocity, and K_m is a constant) developed by Michaelis and Menten [1913] and applied to the initial velocities of sucrose hydrolysis by yeast invertase. The constant, K_m , is a characteristic of the enzyme, but for the action of wheat protease on edestin (Fig. 3) no constant values were obtained. This is perhaps not surprising since the degradation of edestin is probably not accomplished by a single reaction but rather by a series of consecutive reactions, and as a consequence no single reaction constant can be expected to apply. Numerically, K_m should be equal to the substrate concentration at which half the limiting velocity of action is reached. For wheat protease this concentration is 0.5% edestin (Fig. 3), a value which may be compared with those reported by Northrop [1920: 1922] for the action of trypsin on caseinogen and gelatin (0.75 and 0.5%), and for the action of pepsin on ovalbumin (4.5%). No other data appear to have been published for proteolytic enzymes although numerous figures are available for other enzyme reactions. From Fig. 3 the maximum velocity of action of wheat protease on edestin appears to be reached at approximately 2% substrate concentration, whilst from 2 to 4.3% edestin the velocity of action is reasonably constant. No falling off is to be observed, although in the case of certain enzymes, notably that of yeast sucrase [Nelson and Schubert, 1928], the decrease in rate of reaction with increase in substrate concentration beyond the maximum point is quite pronounced and immediate. It was found impracticable to investigate higher substrate concentration than 4.3% edestin (Fig. 3).

The destruction of wheat protease by heat appears to follow a normal course, the critical inactivation temperature at p_H 6.0 being about 54° (Fig. 5).

This critical temperature may vary, however, according to the p_H value adopted at the time of heating. Arrhenius [1907] gives a critical temperature of 65° for both trypsin and pepsin and 46° for rennin. Euler and Laurin [1919] found 59° for invertase at p_H 4.0 and Lüers and Wasmund [1922] 57° for malt amylase.

With regard to the inhibitory effect of chloride ions on wheat protease it seems clear from Fig. 7 that the phenomenon is caused by a decrease in the solubility of the edestin with increasing NaCl concentration. The precipitation of the substrate at concentrations of NaCl corresponding to 0.2 and 0.3 *M* doubtless removed the edestin from the sphere of activity of the enzyme. On the other hand, a similar precipitation of edestin was observed in several of the reaction mixtures made up with acetate buffer alone, particularly at about p_H 5, without an equivalent reduction of activity (Fig. 1).

The dipeptidase of wheat extract, although relatively unstable, is quite an active enzyme, especially with leucylglycine as substrate. Incidentally this instability renders it possible to obtain a dipeptidase-free preparation simply by keeping the extract for 4–5 days at 18° under toluene; this has no serious effect upon the activity of the protease. The dipeptidase induces hydrolysis of leucylglycine much more readily than hydrolysis of glycylglycine. In 3 hours at optimum p_H hydrolysis of glycylglycine is only 13% that of leucylglycine (Figs. 8 and 9). It will be observed that the control curve (Fig. 8) has itself an optimum at about p_H 7.5, doubtless the optimum for dipeptidase activity on those dipeptides which have found their way into the enzyme extract.

The separate identities of protease and dipeptidase are clearly established, firstly, by the great difference in stability between the two and secondly, by the fact that at the p_H at which protease exerts its optimum activity on edestin the dipeptidase does not attack leucylglycine and *vice versa*.

SUMMARY.

1. The aqueous extract of sprouted wheat contains a protease which shows an optimum p_H of 4.1 for decomposition of edestin at 40° .
2. The protease requires a minimum concentration of about 2% edestin for maximum rate of activity at 40° . The data reveal no constant value for the Michaelis constant, K_m . The substrate concentration at which half the maximum reaction velocity is reached corresponds to 0.5% edestin. The course of reaction in the early stages appears to follow the Schutz-Arrhenius rule.
3. The critical inactivation temperature is 54° at p_H 6.0.
4. The protease is spontaneously inactivated on standing at 18° with toluene. The inactivation follows the course of a unimolecular reaction. The activity falls to half the original value in about 18 days.
5. The protease activity is considerably reduced by citrate and chloride ions, owing probably to precipitation of substrate.
6. The aqueous extract contains a dipeptidase which attacks leucylglycine optimally at p_H 7.3 and glycylglycine at p_H 7.9.
7. The dipeptidase is unstable in aqueous solution, all activity being lost in 5 days at 18° in the presence of toluene.

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LXXXI. THE ASSAY AND THE EFFECT OF TESTOSTERONE ON RATS COMPARED WITH THOSE OF OTHER SEXUAL HORMONES.

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LAQUEUR and his co-workers [David, 1935; David *et al.*, 1935; Laqueur *et al.*, 1935] isolated testosterone from the testes of the bull. Ruzicka and Wettstein [1935; Wettstein, 1935] and Butenandt and Hanisch [1935] prepared this compound artificially. The natural and artificial preparations were similar in all respects, one capon unit being contained in about 10γ and the effect on the seminal vesicles of castrated rats being greater than that on the prostate. According to Tschopp [1935; Ruzicka and Wettstein, 1935, p. 1270] 10γ of testosterone injected into castrated rats for 10 days brought about an increase in the size of the seminal vesicles of about 300% and of the prostate of about 113%.

Laqueur *et al.* found that the effect of testosterone on the seminal vesicles and prostate of castrated rats could be doubled or even more than doubled, if a physiologically indifferent, impure extract, prepared from the tissues of animals or plants were injected simultaneously. These observations were corroborated by Tschopp [Ruzicka, Wettstein and Kagi, 1935], who [Ruzicka, Goldberg and Rosenberg, 1935] also compared the various artificial male sexual hormones prepared by Ruzicka and his co-workers. In the same paper Ruzicka *et al.* pointed out the correlation between the chemical constitutions of the hormones and their selective effects in birds and rats.

The following experiments were performed with artificial testosterone prepared by Ruzicka and kindly supplied to us by Messrs Ciba Ltd. The testosterone was dissolved in olive oil and the daily dose was injected in half doses (0.1 ml. of oily solution) twice a day. Two series of experiments were made, one of 7 days' duration, in which the assay of testosterone was performed and a second of 23 days' duration, in which a study was made of the effects of prolonged injections. For economy of space Tables I-V showing the results have been much compressed.

I. THE ASSAY OF TESTOSTERONE.

Assay was performed on 80 rats belonging to 17 litters, the technique used being the same as that described in our previous papers with the following modification. We usually divide each litter into two or three groups, each containing 2 or more rats. A general average percentage change for each dose is then calculated from the percentage changes obtained in each of the groups injected with that particular dose. In the assay of gonadotropic hormone [Korenchevsky *et al.*, 1935, 2, p. 2523] some of the litters were used for an assay made in the way described above, while the rest of the litters were divided so that one rat of each litter was placed in each group and a general average of the weights of the organs obtained for each dose. The percentage changes were then

calculated from these general average weights. The results obtained by the two methods differed very little.

In this assay therefore we tried to simplify the technique still further, in order to economise the number of rats used in the control and injected groups. Two or three litters of the same age and weight were used as if they were one litter, the rats of these litters being distributed evenly in the various groups. Not less than 6 and generally 7 rats were used for each "dose-group". Since preliminary experiments showed that one rat unit was contained in about 8.3 γ , this amount was chosen as the smallest dose, most of the other doses being simple multiples thereof. There were more rats (13) in the control than in any other group, since the former contained 1 rat from most of the litters used in the experiment. The weights of the seminal vesicles and prostates of the uninjected control rats used in our previous assays of sexual hormones [*e.g.* Korenchevsky and Dennison, 1935, 1, 2; Korenchevsky *et al.*, 1935, 1] approximated closely to the averages obtained for these weights in the control group in the present

Table 1. *The effect of 7 days' injection of testosterone on sexual organs, fat and on the gain in body weight of male castrated rats.*

Average actual weights of organs and the average percentage change in weight (actual and per unit of body weight) after injection.												
Organ	Weights	Control rats	Rats injected with (γ)									
			8.3	16.7	33.3	67	125	167	334	500	1000	1410
A. Average weights												
Seminal vesicles (mg.)	Actual	10	16	21	26	28	35	41	57	54	68	87
Prostate (mg.)	Actual	54	76	86	97	102	123	137	187	173	196	230
Prostate with seminal vesicles (mg.)	Actual	64	92	107	123	130	158	178	241	227	261	217
Preputial glands (mg.)	Actual	54	53	57	63	64	82	82	89	96	100	127
Thymus (mg.)	Actual	680	723	710	632	481	569	511	433	558	569	575
Fat (g.)	Actual	8.1	7.4	7.9	7.3	7.5	7.3	6.4	6.9	8.7	6.9	7.7
B. Percentage change in weight												
Seminal vesicles	Actual	—	60	+110	+160	+180	+250	+310	+470	+440	+580	+770
	Per unit of body weight	—	+75	+100	+163	+187	+262	+312	+450	+412	+537	+700
Prostate	Actual	—	+41	+59	+80	+89	+128	+151	+246	+220	+263	+326
	Per unit of body weight	—	+49	+56	+77	+130	+123	+161	+244	+205	+247	+293
Prostate and seminal vesicles	Actual	—	+44	+67	+92	+103	+147	+181	+281	+255	+313	+339
	Per unit of body weight	—	+14	+63	+90	+139	+115	+181	+276	+237	+292	+357
Penis	Actual	—	+27	+58	+52	+51	+93	+92	+121	+118	+146	+149
	Per unit of body weight	—	+35	+51	+49	+54	+88	+97	+116	+107	+131	+128
Preputial glands	Actual	—	+2	+6	+17	+19	+52	+52	+65	+78	+102	+135
	Per unit of body weight	—	+5	+2	+16	+23	+49	+58	+65	+74	+93	+121
Thymus	Actual	—	+6	+4	+7	+20	+16	+25	+36	+18	+16	+15
	Per unit of body weight	—	+13	+0	+9	+27	+19	+24	+38	+22	+21	+23
Fat	Actual	—	+9	+2	+10	+7	+10	+21	+15	+7	+15	+5
	Per unit of body weight	—	+10	+10	+10	+2	+10	+22	+16	+2	+21	+11
Gain in body weight		—	+7	+19	+22	+41	+39	+30	+11	+48	+26	+15

experiment. This indicates that in future assays on castrated rats the control group may be dispensed with, provided that the experiments are carried out with the same stock of rats, bred and fed under the same conditions and that the "standard" weights of the sexual organs of these rats at special periods of time after castration are well established.

Sexual organs. The results of the present experiments are summarised in Table I and represented by the curves (Figs. 1, 2, 3 and 4). In spite of the variation between the individual figures, deviations from the average weight

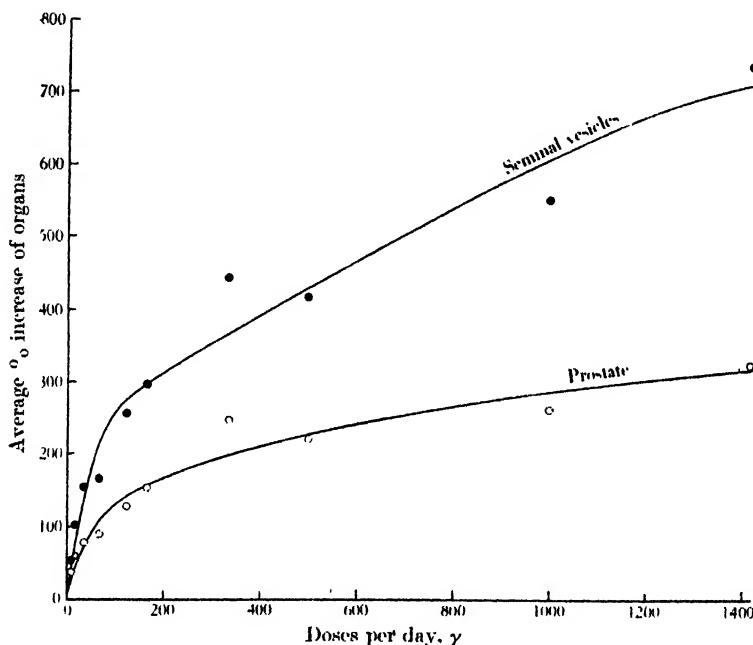


Fig. 1. Relation between dose of testosterone and percentage increase in actual weight of seminal vesicles and prostate.

exceed 25 % only in 3 cases out of 80 in the actual weight of the prostate and in 6 out of 80 in the weight of the prostate per 200 g. of body weight. In these nine variations, the deviation is <27 % in 4 cases and <35 % in another 4; in 1 case only is there a deviation of 57 %. In the seminal vesicles the number of deviations greater than 25 % is 8 out of 160 (80 actual and 80 per unit of body weight). Of these 3 do not exceed 27 %, 4 do not exceed 32 % and in 1 case only the deviation is 40 %.

The percentage increase in the weights of the seminal vesicles and prostate shows a sharp rise with the small doses, followed by a flattening of the curves with the larger doses, which is typical of this hormone. The larger response of the seminal vesicles as compared with the prostate is also characteristic. There is no direct proportion between dose and effect such as was seen within a certain range, for example, with androsterone and androsterone-diol.

One rat unit (minimum dose producing 40 % increase in the weight of the prostate) is contained in 8 γ , calculated from the actual weights; and in about 7 γ calculated from the weight per unit of body weight. The percentage increases in the actual weights of the seminal vesicles and penis corresponding to these

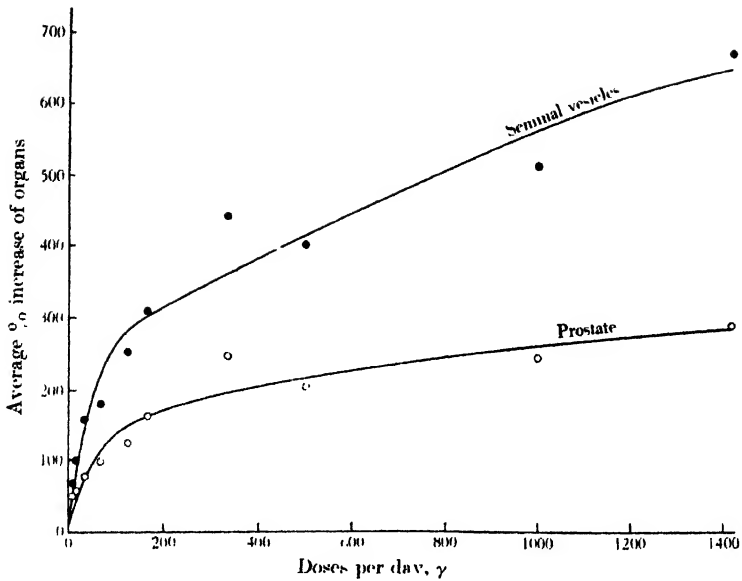


Fig. 2. Relation between dose of testosterone and percentage increase in weight of seminal vesicles and prostate calculated per unit of body weight.

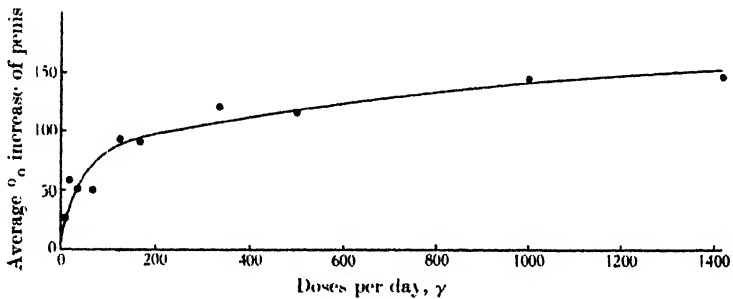


Fig. 3. Relation between dose of testosterone and percentage increase in actual weight of penis.

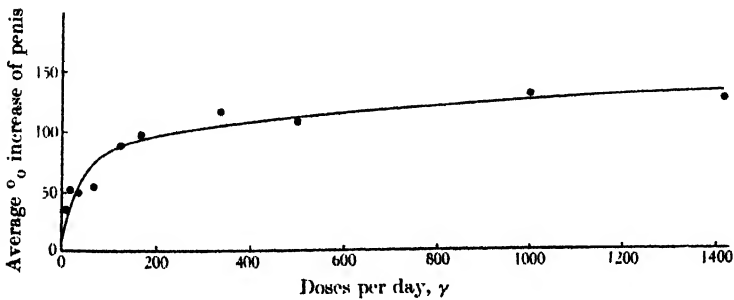


Fig. 4. Relation between dose of testosterone and percentage increase in weight of penis calculated per unit of body weight.

doses are about 60 and 27 % and per unit of body weight 61 and 29 % respectively.

As we have stated before, the weight of the preputial glands in both normal and castrated uninjected rats always shows greater variation than is shown by other sexual organs. As can be seen from Table I doses of 8–16 γ have no definite effect, the changes being too small to be considered. The dose 33 γ has a small definite effect, after which the curve rises more slowly than in the case of the penis but with the highest dose reaches nearly the same height as that reached by the penis.

Thymus, deposition of fat and gain in weight. As can be seen from Table I, even after only 7 days of injection the normal response of the thymus is obtained, i.e. the rate of involution of the thymus, delayed by castration, returns towards normal after the injection. The effect however is comparatively small though definite with doses of 67 γ and more.

The gain in weight (Table I), though only slightly greater in the injected rats, occurs in most cases and with all the doses used. This cannot be explained by an increased deposition of fat, which is in most cases less than that of the control uninjected rats (Table I).

Statistical discussion of the data.

By J. M. C. Scott.

Curves. Curves were fitted to the data by least squares. The system of curves employed was a modification of that described previously [Korenchevsky and Dennison, 1935, 2, p. 2128].

Probable errors. It was clear from the data that the chance fluctuations of the weight of a given organ are not independent of the dose but become greater as the dose is increased. The hypothesis that the probable error of the weight of

Table II. *Statistical summary of results of the assay experiments.*

1 Organ and basis of calculation	J. M. SCOTT.	
	II Probable error for weight of organ (mean of 6 rats)	III Coefficient for probable error for percentage increase in weight of organs
Seminal vesicles (actual weight)	± 4.8	5.8
Seminal vesicles (per unit of body weight)	± 4.2	5.0
Prostate (actual weight)	± 3.8	4.6
Prostate (per unit of body weight)	± 4.1	5.0
Penis (actual weight)	± 3.6	4.3
Penis (per unit of body weight)	± 3.9	4.7

the organ increased proportionally to the weight itself was tested and found to be well supported by the data; the figures in col. II give this probable error as a percentage of the mean weight of the organ for a mean of 6 rats.

The most important results are the percentage increases due to the hormone, and these are affected by any variation in the 13 controls. The figures in col. III take this into account; as they stand they are not probable errors, owing to the variation increasing with the weight of the organ, but they must be increased by the "percentage increase" to which they apply.

For example, with a dose of 33.3 γ the actual weight of the prostate (see

Table I) showed a percentage increase of 80 %. Therefore the probable error in this case will be:

4.6	...	Table II, col. III.
3.7	...	80 % of 4.6.
8.3	...	Probable error required.

In the same way, for a dose of 167 γ which gives 154% increase in actual weight of prostate, we obtain a probable error of ± 11.7 . For the largest dose used 1410 γ , giving 326 % increase in actual weight of prostate, the probable error will be ± 19.6 .

II. PROLONGED PERIOD OF INJECTION (23 DAYS) OF TESTOSTERONE INTO CASTRATED MALES.

In long-duration experiments the relative fluctuations in the weights of the organs are smaller than in the 7-day experiments. Fewer animals therefore were used in these experiments. 29 rats belonging to 5 litters were distributed into 8 groups as shown in Table III. In 4 litters 1 or 2 rats of each litter were left

Table III. *Effect of prolonged injections of sexual hormones on male rats.*

The average actual weights of organs of castrated rats injected with sexual hormones compared with those of the organs of normal and castrated litter-mates injected with oil only.

	I	II	III	IV	V	VI	VII	VIII
			Castrated rats injected with (γ)					
	Normal control rats	Cas- trated control rats	Testosterone				Testost. 167 + androst. 450	Testost. 500 + androst. 450
Organ			33	167	500	1410		
Sem. vesicles (mg.)	999	10	29	81	180	276	98	199
Prostate (mg.)	1250	63	106	230	367	554	295	454
Penis (mg.)	328	79	159	208	243	277	226	256
Preputial gland (mg.)	144	65	65	120	129	185	158	161
Adrenals (mg.)	60	80	78	65	65	60	73	63
Thyroid (mg.)	25	22	23	27	26	30	24	24
Hypophysis (mg.)	10.8	12.6	12.4	14.1	13.3	15.5	14.2	14.2
Thymus (mg.)	315	560	474	500	409	382	548	420
Liver (g.)	13.6	10.3	10.8	12.9	12.6	13.8	13.0	13.6
Kidney (g.)	2.31	1.72	1.83	2.01	1.98	2.22	2.12	2.26
Heart (mg.)	960	795	989	976	919	1023	975	1092
Fat (g.)	14.5	17.2	16.5	15.3	17.1	17.2	17.1	18.9
Final body wt. (g.)	367	320	315	356	348	377	359	380
Gain in body wt. (g.)	80	64	69	85	85	92	78	100

Note. Bulb of penis cut off.

normal while the rest were castrated. These unoperated litter-mates form a control group of normal rats (col. I, Table III), with which to compare the castrated control rats (col. II) and the rats injected with the hormones (cols. III-VIII). The rats of both the normal and castrated control groups (I and II) were injected with 0.2 ml. daily of pure olive oil, since 0.2 ml. daily of a solution of the hormone in olive oil was injected into the rats of Groups III to VIII.

The average final age of the rats was 98 days, the injections being started 51 days after castration and carried on for 23 days. The number of rats in each group is also given in Table III. The technique used was the same as that given in our previous papers, except that the penis was weighed without the lower bulbous part, by which it is attached to the pelvic bones. This lower part was cut off after fixation in Bouin's fixative at the well defined point where the penis

begins to enlarge into the bulbous part. Our experiments show that this does not affect the percentage change in weight of the penis, but it does make the weighing of this organ more accurate.

Sexual organs. The experiments were carried out on rats a long time after they had been castrated and were therefore "recovery" experiments. In no case did the injection of testosterone restore the normal weight of the seminal vesicles, prostate or penis, although the largest daily dose used (1410 γ) contained about 176 rat units (as defined by us) or about 141 capon units. The weight of the preputial glands however not only returned to normal but, with the highest dose, exceeded the normal average.

The addition of a small dose of androsterone, although improving the degree of recovery of the sexual organs, did not give even a summation result. After the injection of 0.45 g. androsterone for 22 days [Korenchevsky *et al.*, 1935, 3] the average weights of the sexual organs were: seminal vesicles 41 mg., prostate 210 mg., penis 209 mg. and preputial glands 115 mg. If these figures are compared with the corresponding figures in Table III, cols. IV and V (testosterone alone) and cols. VII and VIII (testosterone + androsterone), it is seen that the addition of androsterone brought about a very small increase in the "recovery" effect on the seminal vesicles, prostate and penis, causing a return to the normal weight only in the case of the preputial glands.

Adrenals. As always in castrated animals, the adrenals of control castrated rats were enlarged. Testosterone injected in doses of 167 γ or more caused a return towards the normal weight and with the highest dose the normal weight was obtained. This is shown both by the actual figures and the figures per unit of body weight (Table III).

Thyroids. The small changes in the weights of the thyroids may be significant, since they occurred with all doses, although the changes were less pronounced when calculated per unit of body weight. Histological investigation and repetition of the experiments will give a decisive answer. The addition of androsterone did not increase the "recovery" effect.

Hypophysis. These glands were fixed in formalin, since Bouin's solution is not suitable if the glands are to be used for histological investigation. The weights after formalin fixation are not so close to the original weights as after Bouin's fixation. In the castrated injected animals however as before (see our previous papers), hypertrophy of these glands was seen, which surprisingly was found to persist or even appeared to increase (shown more especially in the actual figures) after testosterone injections both alone and with androsterone.

Thymus. The usual effect of delay in the rate of involution of the thymus was seen in the castrated animals and a return towards but not to normal was obtained after injection of testosterone. The "recovery" effect of testosterone however does not appear to be so complete as with large doses of androsterone or diol [Korenchevsky *et al.*, 1935, 3; Table II, p. 2538].

Liver, kidneys and heart. The usual decrease in the weight of these organs was seen in the castrated animals (col. II) as compared with the normal animals (col. I). The injection of testosterone, alone or with androsterone restored the normal weight, even in the case of the heart which sometimes exceeded the normal value.

Deposition of fat and gain in body weight. The deposition of fat, as in our previous experiments, increased (but only to a slight extent) in the castrated control rats as compared with the normal litter-mates. The injections produced no definite effect (Table III). The injections however definitely increased the gain in body weight, which is decreased in castrated animals. Large doses of

testosterone, alone or with androsterone, even increased the gain in body weight above that of normal rats. Since there was no simultaneous increase in the deposition of fat (except in the actual figures for the injection of 500 γ testosterone and 450 γ androsterone, col. VIII), it may be concluded that this increased gain in body weight was due to growth of the other tissues and cannot be explained by an increased deposition of fat.

III. THE EFFECT ON MALE RATS OF THE ADDITION OF OESTRONE OR OESTRADIOL TO TESTOSTERONE INJECTIONS.

Experiments were performed on 20 rats belonging to 4 litters. The rats of each litter were evenly distributed into 4 groups each containing 5 rats. The control group in these experiments was injected with 33 γ testosterone only:

Table IV. *Effect of the simultaneous prolonged injection of testosterone and oestrone or oestradiol on the weight of the organs of male castrated rats.*

Organ	Groups injected with	Average actual weights	
		Weight	Percentage change compared with testosterone-injected rats
Seminal vesicles (mg.)	Testosterone	33	—
	Testosterone + oestrone	40	+ 21
	Testosterone + oestradiol	54	+ 64
	Oestradiol	25	- 24
Prostate (mg.)	Testosterone	107	-
	Testosterone + oestrone	129	+ 21
	Testosterone + oestradiol	138	+ 29
	Oestradiol	69	- 36
Penis (mg.)	Testosterone	148	—
	Testosterone + oestrone	149	+ 1
	Testosterone + oestradiol	144	- 3
	Oestradiol	74	- 50
Preputial glands (mg.)	Testosterone	67	—
	Testosterone + oestrone	76	+ 12
	Testosterone + oestradiol	87	+ 30
	Oestradiol	54	- 19
Hypophysis (mg.)	Testosterone	11.4	-
	Testosterone + oestrone	13.3	+ 17
	Testosterone + oestradiol	12.4	+ 9
	Oestradiol	13.4	+ 18
Thymus (mg.)	Testosterone	537	—
	Testosterone + oestrone	456	- 15
	Testosterone + oestradiol	467	- 13
	Oestradiol	447	- 17
Liver (g.)	Testosterone	10.09	—
	Testosterone + oestrone	9.41	- 7
	Testosterone + oestradiol	8.48	- 16
	Oestradiol	7.83	- 22
Heart (mg.)	Testosterone	870	—
	Testosterone + oestrone	773	- 11
	Testosterone + oestradiol	719	- 17
	Oestradiol	699	- 20
Gain in body weight (g.)	Testosterone	100	—
	Testosterone + oestrone	75	- 25
	Testosterone + oestradiol	53	- 47
	Oestradiol	53	- 47

Note. The hormones were injected in the following doses, oestrone 6 γ and oestradiol 20 γ , three times per week; testosterone 33 γ daily. Bulb of penis cut off.

group II was injected with 33 γ of testosterone and in addition with 6 γ oestrone; group III with 33 γ of testosterone and 20 γ of oestradiol and group IV with 20 γ of oestradiol only. Testosterone was injected twice daily and oestrone and oestradiol were injected three times a week separately in another area of the subcutaneous tissue. The rats were castrated before reaching sexual maturity, the average final age being 74 days. On the average, the injections were started 28 days after castration and continued for a period of 23 days. The results are summarised in Table IV.

Sexual organs. The addition of oestrone or of oestradiol had only a small co-operative effect on the weight of atrophied seminal vesicles, prostate and preputial glands and no effect on the penis. The co-operative effect was more marked with oestradiol, which was not only injected in larger amount than oestrone, but has a greater effect on the seminal vesicles of castrated rats when injected in the same amount as oestrone, the effect of oestradiol being about twice that of oestrone [David *et al.*, 1934; David, De Yongh and Laqueur, 1935]. An increase in the dose of oestrone used would probably give a co-operative effect similar to that given by oestradiol. Oestradiol alone, as has been described by David, Freud and De Yongh, has a stimulating effect on the seminal vesicles and prostate (the authors obtained on the average 55 % increase in the weight of the prostate and 129 % increase in that of the seminal vesicles after injections of 0.2-6 γ daily for 5-6 days). The increase in the weight of the seminal vesicles was attributed to hypertrophy of the muscular layer. If the weights of the sexual organs of the rats in the present experiments are compared with those of uninjected castrated rats of similar age used in previous experiments, it is found that oestradiol injected during 23 days caused an increase of 108-150 % in the weight of the seminal vesicles and of 19-28 % in the weight of the prostate. The weight of the preputial glands increased in only two (by about 20 %) out of 5 rats and there was no effect on the penis. The effect was much less than that brought about by 33 γ of testosterone (Table III).

Hypophysis. Oestradiol, alone or with testosterone, caused a slight increase in the hypertrophy of the already hypertrophied hypophysis.

Thymus. The rate of involution of the thymus, which is delayed in castrated rats, was slightly increased by oestradiol either alone or when added to testosterone injections.

Liver and heart. There was a quite definite decrease in the actual weights of these organs after the injection of oestradiol alone or with testosterone, which decrease disappeared or became insignificant when the results were calculated per unit of body weight. This shows that, after treatment with female hormone, the changes in body weight are related to the condition of these organs.

Deposition of fat and gain in body weight. The deposition of fat, as judged by changes in the amount of intra-abdominal fat, decreased by about 24 % when oestradiol, alone or with testosterone, was injected. The effect of oestrone was less noticeable, the decrease with this hormone being only about 11 %. These changes are small in view of the normally large variations. Taken however in conjunction with the quite definite decrease in the gain in body weight (Table IV) which is produced by oestradiol and oestrone these figures become significant. There is therefore an antagonistic action on the gain in body weight between testosterone and oestrone.

The adrenals, thyroid, kidneys and spleen did not show any definite changes in weight.

IV. THE EFFECT ON OVARIECTOMISED RATS OF TESTOSTERONE INJECTED ALONE OR SIMULTANEOUSLY WITH OESTRONE.

Experiments were performed on 42 rats, 7 normal and 35 ovariectomised before sexual maturity. These rats are represented in Table V in two groups, according to age, one group on the average 99 days old and the second group 56 days old. The rats belonged to 7 litters with the exception of 8 (out of 11) spayed control

Table V. *Effect of sexual hormones on female rats.*

The average actual weights of the organs of spayed rats injected with sexual hormones as compared with those of the organs of normal or spayed control rats injected with oil only.

	I	II	III	IV	V	VI	VII	VIII	IX	X
	Spayed rats injected with (γ)									
Organ	Normal control rats	Spayed control rats	Oestrone 6	Testosterone			Oestrone 6 + Testost. 167	Oestrone 6 + Testost. 5	Oestrone 6 + Testost. 700	Average age of rats, days
Uterus (mg.)	415	39	165	167	500	700	235	229	290	99
	—	32	—	—	75	121	—	—	—	56
Cervix of uterus (mg.)	117	17	59	25	—	—	94	98	104	99
	—	10	—	—	22	50	—	—	—	56
Vagina (mg.)	254	121	228	184	—	—	267	252	284	99
	—	130	—	—	214	225	—	—	—	56
Fem. preputial glands (mg.)	105	84	90	140	—	—	148	162	207	99
	—	100	—	—	124	119	—	—	—	56
Adrenals (mg.)	81	82	82	71	—	—	83	80	79	99
	—	94	—	—	82	61	—	—	—	56
Thyroids (mg.)	22	20	22	25	—	—	23	24	25	99
	—	18	—	—	20	21	—	—	—	56
Hypophysis (mg.)	13.8	12.1	12.6	11.2	—	—	12.2	14.3	12.5	99
	—	12.0	—	—	10.6	9.6	—	—	—	56
Thymus (mg.)	279	511	331	388	—	—	315	307	283	99
	—	672	—	—	458	339	—	—	—	56
Average final body weight (g.)	230	203	281	272	—	—	270	282	278	99
	—	204	—	—	202	203	—	—	—	56

Note added March 9th, 1936. The experiment with injection of testosterone into ovariectomised rats was repeated on 18 rats and similar results were obtained which will be published elsewhere.

rats in the second group, which were taken from our previous experiments. The number of normal control litter-mates was increased in the present experiment, in order to determine how far the injections restored the normal condition. The control rats were injected with pure olive oil. In order to ensure that there should be no regeneration of ovarian tissue from ovarian cells left after the operation, the ovaries were removed with the surrounding fat tissue and part of the horn. The length of the piece of horn removed varied and in any case shortened the horns of the spayed rats as compared with those of normal rats. Therefore in order to avoid a fallacious comparison with the uterus having horns of normal length, in this experiment we weighed not only the uterus with horns, but also the cervix of the uterus separately. These latter weights seem to give a more accurate representation of changes in the uterus of spayed rats compared with normal animals. Another difficulty in standardising the "normal" weight in the case of the female sexual organs, especially of the uterus, lies in the cyclic changes. The much greater "oestrus" weight of the uterus (obtained in one of the rats) was excluded from the average figure given in Table V (col. I). Theoretically, it would be expected however that oestrone would produce a uterus having an "oestrus" weight, which with the doses used we never

actually obtained. Testosterone was injected daily and oestrone three times a week. The period of injection of testosterone alone was 22 days and of oestrone alone or with testosterone 21 days.

Sexual organs. Testosterone injected alone (cols. IV-VI) caused hypertrophy of the atrophied sexual organs. The hypertrophy however was not sufficient (except in the case of the preputial glands) to represent complete recovery, nor in the case of the uterus was it equal to that produced by oestrone (col. III), though after injections of 700 γ daily of testosterone the vagina weighed about as much as after the injection of oestrone.

In the dose used, oestrone alone was unable to restore the normal condition of the sexual organs of ovariectomised rats. After simultaneous injections of oestrone and testosterone, recovery to the normal weight was complete in the case of the vagina but incomplete in the case of the uterus, although the weight of this organ was considerably greater than after injections of oestrone alone (especially if judged by the changes in the cervix of the uterus), when the largest dose of testosterone was added (col. IX). The weight of the preputial glands after the simultaneous injection of the hormones exceeded the normal weight.

Thus the co-operative activity of testosterone and oestrone in restoring the atrophied female sexual organs towards or in some cases, to the normal weight was clearly seen in all the experiments.

Other organs. The effect of ovariectomy on the weight of most of the organs, which are definitely affected in males by castration (adrenals, hypophysis, liver, kidney, heart, deposition of fat) could not be clearly seen, though the delay in the rate of involution of the thymus was quite definite (Table V). We do not however give definite conclusions with regard to the effect of ovariectomy on these organs, since for this a larger number of experiments would be necessary, especially considering the increase in weight of some of the organs of normal rats, which occurs during oestrus (*i.e.* adrenals, thyroids, hypophysis [Andersen and Kennedy, 1932: 1933, 1, 2]). According to Andersen and Kennedy, in rats spayed before sexual maturity and killed about 65 days after, there is no change in the weight of the adrenals as compared with that of rats in dioestrus; the thyroids are below normal weight, while the weight of the hypophysis lies between that of the normal controls in oestrus and those in dioestrus.

The figures for these organs given in cols. I and II of Table V are in agreement with the conclusions of Andersen and Kennedy.

The effect of the injection of testosterone alone was slight, but occurred in some of the organs of all rats, the weights of the adrenals and hypophysis being decreased and of the thyroids being increased. The addition of oestrone to testosterone injections prevented the development of this effect on the adrenals and hypophysis.

Injections of oestrone and of testosterone separately or simultaneously restored the rate of involution of the thymus to or towards normal.

The body weight and the deposition of fat were not definitely affected by the injections, but the average body weight was increased by ovariectomy (col. II) compared with that of the normal control rats (col. I), in spite of the absence of an increase in the deposition of fat.

V. DISCUSSION.

It is only possible to estimate the activity of a hormone preparation and to compare it with that of other preparations, when all the results both of experiments of short and of long duration are taken into consideration. In the following

an attempt has been made to make such a comparison of testosterone with androsterone, androsterone-diol, gonadotropic hormone and the manifold and partly undefined factors which produce maturation of the sexual organs in normal animals.

Until the histological investigation has been completed, we cannot say how far the changes obtained in the weights of the organs after injections represent a true recovery. The results so far obtained indicate that the histological changes in the female sexual organs after the injection of testosterone are similar to those previously described [Korenchevsky and Dennison, 1936], which were obtained after the injection of androsterone-diol, *i.e.* they are similar to some of the changes produced by progesterone or observed during pregnancy.

Effect on castrated males.

On the method of assay. The statistical interpretation of the assay results by Mr Scott shows that the probable error for percentage changes of weight of organs increases with the weight of the organs. Therefore in the assayable part of the curve (*i.e.* from 8 to 334 γ , from which point the curve flattens sharply) the probable error for prostate gradually rises from ± 6.5 to ± 15.9 . Provided that not less than 6 rats are used for each group-dose, the results of assay with such errors should be considered as satisfactory, in spite of the simplification of the method introduced.

Using Mr Scott's coefficient the probable error for seminal vesicles is found to be greater than that for prostate. This supports our repeated statement that prostate is more suitable for assay experiments than seminal vesicles. However, the changes in seminal vesicles and penis are suitable for corroboration of the results obtained with prostate.

The rat unit. This unit, as defined in our previous papers (the minimum dose producing 40% increase in the weight of the prostate) only represents the smallest active dose of the hormone. As judged by changes in the actual weight of the prostate, one rat unit is contained in 8 γ of testosterone (present experiments), 21 γ of androsterone-diol [Korenchevsky and Dennison, 1935, 2] and 170 γ of androsterone [Korenchevsky and Dennison, 1935, 1]. A comparison of the rat units therefore indicates that testosterone has the greatest activity.

Comparison of the curves. The curves representing the percentage increase in weight of the seminal vesicles after the injection of androsterone, androsterone-diol and testosterone are given in Fig. 5, while those for prostate are given in Fig. 6. Because of the low solubility of fat-soluble (f.s.) androsterone-diol in oil the curve continues only to the point representing 350 γ of the hormone. Therefore androsterone-diol is also represented by the curve for water-soluble (w.s.) androsterone-diol (the Li salt of the monosuccinate of this hormone).

Since the curves are different in form, a comparison can only be made with reservations.

The curves for seminal vesicles (Fig. 5) show that fat-soluble and, less closely, water-soluble androsterone-diol are similar to testosterone in their activities and differ from androsterone by a greater effect on the seminal vesicles, the activity of testosterone being greatest.

The curves for prostate (Fig. 6) are nearly identical in the case of fat-soluble androsterone-diol and testosterone. The curve for water-soluble androsterone-diol differs from these, but not greatly. Androsterone curves show the lowest activity for all doses.

Average activity of the hormones. Another method of representing the activity has already been used by us [Korenchevsky and Dennison, 1935, 2, p. 2128;

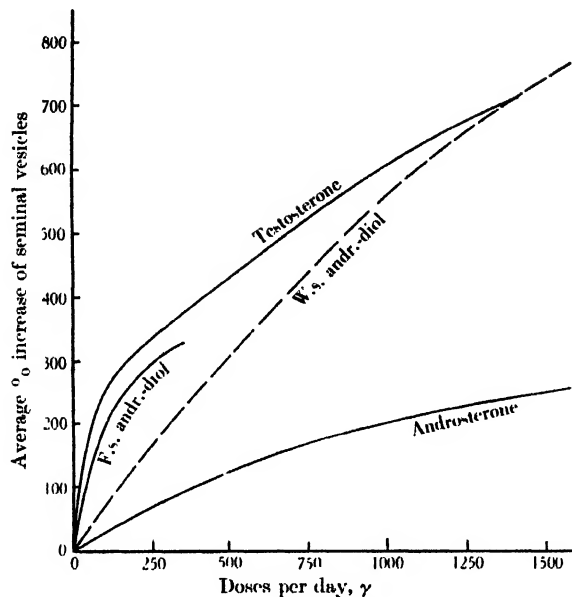


Fig. 5. Comparison of the curves representing relation between percentage increase in the actual weight of seminal vesicles and dose for androsterone, fat-soluble (f.s.) and water-soluble (w.s.) androsterone-diol and testosterone.

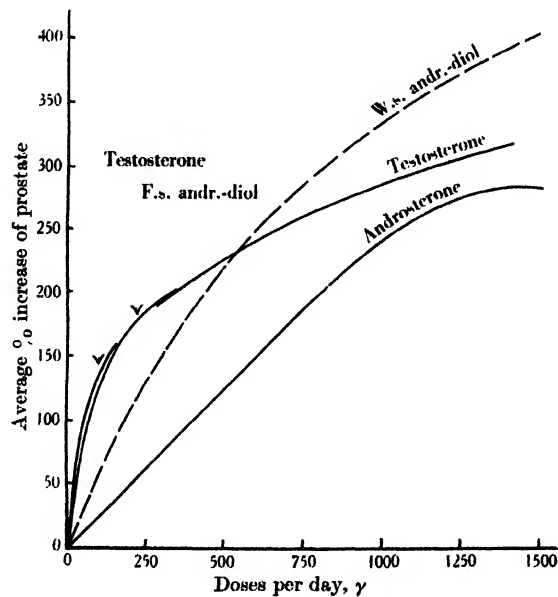


Fig. 6. Comparison of the curves representing relation between percentage increase in the actual weight of prostate and dose for androsterone, f.s. and w.s. androsterone-diol and testosterone. Lower part of the curve for testosterone and the curve for f.s. androsterone-diol are very close, almost coinciding. They are distinguished in the figure by arrows.

Korenchevsky *et al.*, 1935, 1, Table VII, p. 2139]. Within the range of the curve, from the smallest active dose to the dose at which the curve begins to show pronounced flattening, comparison was made of the average percentage increase in the weights of the organs per 100 γ for each dose used. The figures of this comparison, given in Table VI, show that androsterone-diol appears to have the greatest average activity.

Table VI. *Comparison of the average effects of androsterone, androsterone-diol and testosterone (7 days' injections).*

Organs	Weights	Average percentage increase per 100 γ for daily doses		
		200-300 γ andro- sterone	25-200 γ andro- sterone- diol	8-334 γ testo- sterone
Seminal vesicles	Actual	23	223	205
	Per unit of body weight	23	216	206
Prostate	Actual	25	144	106
	Per unit of body weight	24	135	112
Prostate with seminal vesicles	Actual	24	156	122
	Per unit of body weight	23	149	121
Penis	Actual	11	100	66
	Per unit of body weight	10	93	65
Preputial glands	Actual	18	37	28
	Per unit of body weight	18	33	29

Comparison of the qualitative effect. Before forming a judgment about the activity of a hormone it is essential to analyse its specific activity from the point of view of its approximation to the natural biological process, which is partly or wholly controlled by the hormone. It is now clear that the natural development of the sexual organs is controlled by many factors, including various hormones. The activities of these manifold, but as yet not completely discovered, factors can be demonstrated if the weights of the different sexual organs of normal rats at different ages after sexual maturity are compared with the weights of these organs before sexual maturity. It is obvious however that the natural growth of the sexual organs, which develop during a period of some months, can only be compared with experiments in which (1) the effect of the injections develops during a period sufficiently long for the recovery to take place and (2) the doses injected are large enough to compensate the still relatively short period of injection (weeks instead of months) by increasing the dose of the hormone injected.

Our Table VII therefore is divided into two parts: in the first part experiments on the forced development of the sexual organs during a 7-day period of injection of artificial hormones are compared with a 5-day period of injection of gonadotropic hormone [Korenchevsky *et al.*, 1935, 2], which hormone is supposed to bring about normal stimulation of the normal secretion of the testicular hormones in their natural relationship. In the second part of the table the ratios obtained after a long period of injection of artificial hormones (the effect of large and smaller doses being given separately) are compared with the ratio given by naturally developed sexual organs. This "natural" ratio was obtained from the percentage increase of the actual weights of the sexual organs of normal rats 49-123 days old taken from our previous paper [Korenchevsky *et al.*, 1935, 3, Table I, p. 2535] compared with the weights of the organs of immature normal rats 26-27 days old [Korenchevsky *et al.*, 1935, 2, Table I, p. 2523]. The average

Table VII. *Ratio ($\times 100$) of percentage increase in actual weight of prostate to that of seminal vesicles after injection of testosterone compared with this ratio obtained with other hormones or observed during natural growth of the organs in normal rats.*

	Short-period injections		Long-period injections	
	Daily dose (γ)	Ratio prostate to sem. ves.	Daily dose (γ)	Ratio prostate to sem. ves.
Testosterone	8	68	33	36
	16	54	—	—
	33	50	—	—
	67	49	167	37
	125	51	—	—
	167	50	500	28
	334	52	—	—
	500	50	1410	29
	1000	45	—	—
	1410	42	—	—
Testosterone	Average	51	—	—
Gonadotropic hormone	Average	54	—	—
Androsterone-diol	Average	64	175-350	54
			700 or more	24
Androsterone	Average	106	450-2200	126
			3600 or more	55
Natural development in normal rats	—	—	—	25

for all ages of the ratio ($\times 100$) of percentage increase in the weight of the prostate to that of the seminal vesicles was 25.

The ratios given by testosterone are tabulated separately for each dose, whilst for the other hormones only the average ratios are given, since these have been given in detail elsewhere [Korenchevsky *et al.*, 1935, 1, p. 2141]. It is clear from the figures in Table VII that (1) the ratios of the short-period injections show that testosterone and, less closely, androsterone-diol produce development of the sexual organs similar to that produced by gonadotropic hormone and that therefore probably they bring about normal development, whilst androsterone does not do so; (2) the ratios of the long-period injections show a clear-cut difference (as was anticipated and explained above) between the results given by small and large doses, the small dose ratios being closer to those obtained for the short-period injections; (3) the ratios for long-period injections of large doses show a normal or nearly normal development of the sexual organs in the case of androsterone-diol and to a less extent in the case of testosterone, but not in that of androsterone.

The prostate to penis ratio is not included in Table VII as the penis of untreated castrated rats is larger than the penis of the young immature normal rats which were used for comparison with the older rats. This would therefore give percentage increases in the weight of the penis after injections of artificial hormone which would not be comparable with those given by injections of gonadotropic hormone or by natural development. The ratios of these organs in the castrated rats injected with artificial male hormone can however be compared with each other, in which case it is again seen that the development produced by testosterone is similar to that produced by androsterone-diol (ratios 168 and 158 respectively) and is different from that produced by androsterone (ratio 230).

Comparison of the "recovery" experiments on males. In the long-period experiments even the largest doses used (1410 γ =176 rat units) failed to restore

the normal weight of the seminal vesicles, prostate and penis of castrated rats injected for 23 days.

It is difficult to compare these experiments with our previous experiments [Korenchevsky *et al.*, 1935, 3, Table I, p. 2535], in which complete recovery of the prostate was obtained with androsterone and of all the sexual organs with androsterone-diol, since in these earlier experiments the period of injection and the period after castration were different from those of the present experiments. The experiments with water-soluble androsterone-diol (Nos. 19-21) might however be considered to be similar to the present experiments with testosterone. From these it is seen that about 80 rat units (5310 γ) of water-soluble diol brought about complete recovery, whilst 176 rat units of testosterone failed to do so. It would be necessary however to make comparative experiments on litter-mates before coming to definite conclusions on this matter.

If the restorative effects of testosterone on other organs are compared with those obtained with androsterone and androsterone-diol, they are found to be similar in the case of the adrenals, thymus, liver, kidney and heart. The gain in body weight was also found to be improved by all three hormones, though too large doses of water-soluble diol were toxic, as is shown by the decrease in the weight of the liver and in the gain in body weight.

The action of testosterone differed from that of the other two hormones in failing to decrease, even with the largest dose, the hypertrophy of the hypophysis, which with some doses was even increased.

Effect on ovariectomised rats.

A comparison of the figures of Table VI of this paper with Table V of our previous paper on androsterone and androsterone-diol [Korenchevsky *et al.*, 1935, 3, p. 2543] shows that testosterone restored the normal weight of the female sexual organs more completely than androsterone, the change being about the same as that obtained with androsterone-diol.

Whilst the effects on the thymus and hypophysis were similar with all three hormones, the decrease in weight of adrenals and the increase in those of the liver and kidney only occurred after androsterone or androsterone-diol injections. Each of these three male hormones when injected with oestrone showed about equal degrees of co-operative recovery effect on the atrophied female sexual organs (compare Table V of the present paper with the results given in Table VI of the previous paper and those given by the histological data [Korenchevsky and Dennison, 1936]).

Summarising this comparative analysis of these three artificial male hormones it may be concluded that the action of testosterone is similar to that of androsterone-diol, the most important difference between the two substances being the greater activity of the smallest doses of testosterone, *i.e.* in the amount of the substances per rat unit, while in the largest doses used the effect on the sexual organs seemed to be greater with androsterone-diol than with testosterone (long-period experiments).

On X-substance of testosterone.

Experiments on male rats have shown that in the doses used neither addition of androsterone nor of oestrone to testosterone injections was able to give such a large co-operative effect as was produced by the X-substance of Laqueur and his co-workers, only a small co-operative effect being obtained by the addition of the hormones mentioned above.

SUMMARY.

1. Experiments were performed on 5 normal males, 7 normal females, 124 castrated males and 35 ovariectomised rats belonging to 33 litters, in order to investigate the biological properties of artificial testosterone and the co-operative effect of this hormone with oestrone and with oestradiol.

Castrated males.

2. The hormone was assayed by a simplified form of the authors' method. One rat unit was found to be contained in 8γ (from actual weight of prostate increase) or 7γ (from weight per unit of body weight).

3. A relation between the dose of testosterone and the effect on the prostate, seminal vesicles and penis after 7 days of injection was investigated statistically and represented in the form of curves.

4. Prolonged periods of injection (23 days) in the doses used (up to $1410\gamma = 176$ rat units daily) failed to restore the normal weight of the atrophied sexual organs (except in the case of the preputial glands) of rats castrated about 51 days before the beginning of the injections ("recovery" test).

5. After the injections of testosterone the weights of the organs, which were changed by castration, were restored to the normal weights in the case of adrenals, thyroid, liver, kidneys and heart; towards the normal condition in the case of the thymus. There was no change, or an apparent increase in the "castration" hypertrophy of the hypophysis. The gain in body weight also increased to or even exceeded the normal standard value, without definite changes occurring in the deposition of fat; this indicates a favourable stimulation of anabolic processes in the metabolism of organs and tissues.

6. In the doses used, injections of oestradiol alone brought about changes similar to those obtained after injection of oestrone: *i.e.* a considerable increase in the weight of the seminal vesicles (up to 150%) and small increases in the weight of the prostate (up to 28%) and the preputial glands (up to 20%), the penis remaining unchanged. Changes were also observed in the weights of other organs: in the hypophysis (slight increase), in the liver, heart and thymus (decreases). The gain in body weight and deposition of fat were both decreased.

7. Oestrone or oestradiol injected with testosterone showed only a slight co-operative effect on the weight of atrophied seminal vesicles and prostate.

Ovariectomised females.

8. Testosterone injected alone brought about hypertrophy of the atrophied sexual organs towards but not to the normal weight (except in the case of the preputial glands which reached normal weight) and decreased the weight of the thymus.

9. There was a marked co-operative effect between testosterone and oestrone in restoring the atrophied sexual organs to or towards the normal weight. The addition of oestrone to testosterone injections also increased the rate of involution of the thymus (co-operative effect).

10. Thus our previous results with androsterone and androsterone-diol, obtained by the method of weights and corroborated histologically, and also the present results with testosterone, show that the male hormones have some of the important properties of the female hormone: so that, if they are present in the female organism, it would be expected that they would act in co-operation with oestrone in controlling the condition and function of the sexual and of some other important organs in the female.

Comparison of the effects of the hormones.

11. Comparison was made of the biological properties of testosterone, androsterone, androsterone-diol and gonadotropic hormone. The effects of these hormones on the growth of the sexual organs were also compared with the natural growth of these organs in normal rats.

12. Androsterone-diol and testosterone, unlike androsterone, appear to bring about a qualitatively normal development of the male sexual organs, as judged by the ratio of the percentage increase in the weight of the prostate to those of the seminal vesicles and of the penis.

13. Both androsterone-diol and testosterone produce some changes which are similar to those observed after the injection of progesterone and, if injected simultaneously with oestrone, the changes produced are similar to some of those seen during pregnancy.

Grants from the Medical Research Council and from the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish to offer to Prof. L. Ruzicka our deepest gratitude for all the valuable help and information which he has given us during our co-operation in the work on his artificial hormones. We are much indebted to the Department of Statistics of University College and to Mr J. M. C. Scott. We also wish to express gratitude to Dr A. Girard for kindly supplying us with oestrone and oestradiol and to Messrs Ciba Ltd., in particular to Dr K. Miescher, for the generous supply of testosterone for these experiments and of androsterone and androsterone-diol for the previous experiments.

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LXXXII. SOME NOTES ON THE ISOLATION OF OESTRONE AND EQUILIN FROM THE URINE OF PREGNANT MARES.

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LAST year a method was reported [Beall and Marrian, 1934] for the isolation of oestrone from the urine of pregnant mares. By means of a solvent-partition process a crude toluene extract of the acid-hydrolysed pregnancy urine was purified to give a "weak phenol" concentrate containing the extracted oestrone. The ketonic material in this fraction was precipitated from alkaline solution by the use of mercuric hydroxide and the oestrone separated by high-vacuum distillation.

Several modifications in the process have since been introduced. Hydrolysis conditions have been improved, thus increasing greatly the yields of active material which can be removed by the initial toluene extraction; the solvent process has been shortened and the mercuric hydroxide-ketone reaction has been modified so that it is more easily conducted. By this modified process it is possible to obtain high yields of pure oestrone (m.p. 254–256° uncorr.) and also considerable amounts of equilin.

Acid hydrolysis of the urine.

The urine was collected in weekly batches and was concentrated *in vacuo* to about one-quarter of its original volume, all subsequent steps being carried out with such concentrates.

In the previous paper it was pointed out that it is more satisfactory to carry out the acid hydrolysis of mare's urine at room temperature than to boil or to autoclave the acidified material, since the liberated oestrone is readily destroyed in an acid medium at high temperatures. Extremely high yields of active material have been obtained by acidifying the urine concentrates to p_H 0.8–1.0 and keeping them at room temperature for 7–10 days before commencing the toluene extractions. These conditions are the same as those recommended by Curtis [1933] but much higher yields of oestrogenic material have been obtained than previously reported in the literature.

The toluene extracts were processed by the method already described and the oestrogenic materials in the final "weak phenol" concentrates were assayed by the colorimetric method of Cohen and Marrian [1934].

The "weak phenol" concentrates would be expected to contain other phenolic oestrogenic compounds such as equilin, hippulin and equilenin [Girard *et al.*, 1932, 1, 2, 3]. Whilst it was realised that these might also act as chromogens, the colorimetric assays were, for convenience, evaluated in terms of oestrone. It was recognised that such values might not be absolute, but it was

considered that they would, at least, be relative and serve as a basis of comparison for the different batches of urine. In cases where the "weak phenols" were assayed in terms of oestrone by the biological method the values obtained were, within experimental error, in agreement with the colorimetric figures, thus justifying this procedure.

Excretion of oestrin by the pregnant mare.

Urine was collected from a mare throughout the term of pregnancy and, after hydrolysis by the method described in the previous section, colorimetric assays were carried out on the "weak phenol" fraction. The concentration of oestrogenic substances in the urine was too low during the early stages of pregnancy to warrant their isolation. Collections were therefore limited to a period of one week at monthly intervals. As soon as the amounts of active material had risen sufficiently to make their isolation possible, all the urine excreted was collected. The results of the experiment are given in Table I.

Table I. *Oestrin excreted by a mare (number 439).*

Served Aug. 15, 1934, foaled July 14, 1935.

Volume of urine l.	Period of collection	Month of pregnancy	Chromogenic material in "weak phenol" conc. (assayed as oestrone) g.	Chromogenic material per litre unconc. urine mg.
23	Sept. 10-17	1	0.002	Trace
27	Oct. 9-18	2	0.020	0.7
45	Nov. 12-18	3	0.25	5.5
18	Dec. 10-17	4	0.59	33
32	Jan. 7-14	5	2.68	84
118	Jan. 14-Feb. 11	6	9.41	80
100	Feb. 11-Mar. 11	7	10.42	104
77*	Mar. 11-Apr. 22	8	7.98	104
77	Apr. 22-May 20	9	3.52	46
104	May 20-June 15	10	1.97	19
45	June 15-July 1	11	0.22	4.9

* Part of collection lost.

This table shows that the amount of oestrogenic material excreted in the urine starts to rise during the 3rd month of pregnancy, reaches a maximum during the 7th and 8th months and then falls off rapidly so that at term only a relatively small quantity is present. These findings are in agreement with those reported by other workers [Cole and Saunders, 1935; Kober, 1935].

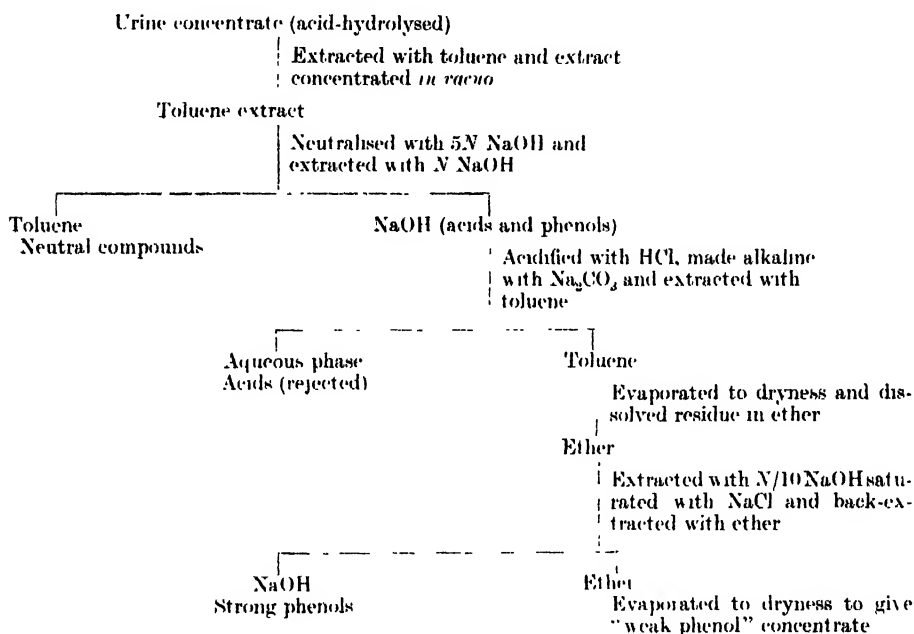
It should be borne in mind that the values in Table I do not represent the total oestrogenic potency of the original urine since 20-30% of the activity is removed in the "strong phenol" fraction. Since the oestrone is concentrated in the "weak phenol" fraction this loss of active material was not investigated.

Modified solvent partition process.

The purification of the crude toluene extract of the acid-hydrolysed urine has been greatly shortened by the adoption of the following procedure.

After concentration of the initial toluene extract to a reasonable volume, sufficient 5*N* NaOH was added to neutralise the acidic substances present, the aqueous phase was removed and the toluene extracted with several portions of *N* NaOH. The combined aqueous extracts were acidified with HCl, made

alkaline with an excess of saturated Na_2CO_3 and re-extracted with toluene. This removed the greater part of the phenolic materials, including the oestrogenic compounds, while the acids remained in the aqueous phase as their sodium salts. The toluene was washed with water, evaporated to dryness and, as in the previously described procedure, the "strong" and "weak" phenols were separated from ether solution by washing with $N/10$ NaOH . It was found that saturation of the NaOH with NaCl prevented the retention of oestrone by the alkaline solution. The extraction process is summarised in Table II.

Table II. *Modified solvent purification process.*

A further purification of the "weak phenol" concentrates has been introduced before the mercury reaction. Vacuum-distillation of the concentrates at 90–100°/0.02–0.03 mm. removed 45–55% of the weight without any loss of oestrogenic material. The residues obtained by this treatment were, in general, dark brown crystalline masses. Some typical results obtained by this procedure are given in Table III.

Table III. *Effect of distillation of "weak phenol" concentrates.*

Original weight of concentrate g.	Weight after distillation g.	% loss of weight	Colorimetric assay of residue (as oestrone) g.	% chromogenic material in distilled residue
3.90	2.07	47	0.72	35
11.01	5.80	47	2.82	49
4.43	2.17	49	1.26	58
4.45	2.09	53	1.50	71
2.93	1.49	49	0.90	60

Modified mercuric hydroxide-ketone reaction.

Interaction of the ketonic material and mercuric hydroxide was brought about, as previously described, by heating in aqueous alkaline solution. After cooling the mercury-ketone complex was precipitated by the addition of ammonium hydroxide and, after standing overnight, the solids were centrifuged out and washed. This procedure was found to be much more satisfactory than that previously described.

The washed complex was hydrolysed by boiling for 15 min. in 15% (by volume) alcoholic HCl and the hot solution was filtered to remove any insoluble mercury salts.

The tedious chloroform-potassium chloride separation for the removal of the mercuric salts from the ketonic materials has been dispensed with. Instead, the excess of HCl in the filtered hydrolysate was neutralised with NH_4OH , the solution being kept just acid to prevent mercuric hydroxide from precipitating. The alcohol was distilled off *in vacuo*, the residue diluted with water to dissolve the inorganic salts, and the granular ketonic material filtered off and washed well with water. To ensure the complete removal of mercuric compounds the washed solid was dissolved in alcohol and treated with H_2S . After filtering out any HgS , the alcohol was removed and the oestrogenic material in the residue was separated by sublimation at $160\text{--}170^\circ/0.02\text{--}0.03$ mm.

Attempts to recrystallise pure oestrone directly from this sublimate met with failure. However, utilising the quinoline reaction described by Butenandt and Westphal [1934] and recrystallising the purified product from 95% alcohol, the pure hormone was obtained.

Isolation of equilin.

In the previous paper it was pointed out that only part of the oestrogenic material of the "weak phenol" concentrates appeared in the mercury-ketone complex. This loss was found to be due mainly to equilin which occurs in the urine in relatively large amounts. It forms a mercury complex precipitated by NH_4OH , which differs from the oestrone complex in that it is soluble in alcohol and so is removed to a great extent in the alcoholic washings.

In order to recover the equilin from these washings they were concentrated to a small volume and the equilin complex was hydrolysed by adding 15% (by volume) HCl and boiling for 15 min. The hydrolysate was treated in a similar manner to the hydrolysate of the mercury-ketone complex and the crude equilin was filtered off. This material was distilled at $160\text{--}170^\circ/0.02\text{--}0.03$ mm. and the equilin (m.p. $238\text{--}239^\circ$) recrystallised from the distillate by the use of 80% alcohol.

Difficulty was encountered in certain batches of urine where the equilin content was higher than usual since in such cases it was not removed completely by the alcoholic washings of the mercury-ammonia precipitate and interfered with the final purification of the oestrone. This trouble was overcome by heating the distilled "weak phenol" concentrate with benzene (10 ml. benzene per g. of concentrate) before carrying out the mercury reaction. On cooling a crystalline precipitate was formed containing 80–90% of the oestrone together with so little impurity that it could be isolated directly by use of the quinoline reaction and subsequent recrystallisation from alcohol. The benzene mother-liquors were taken to dryness, the ketonic and non-ketonic compounds separated by the mercury reaction and the equilin and oestrone isolated from the alcoholic washings and mercury-ketone complex respectively in the manner already outlined.

Summary of the method finally adopted.

The acid-hydrolysed urine was extracted with toluene and the "weak phenols" separated by partition with solvents as outlined in Table II. After vacuum distillation this concentrate was divided into benzene-soluble and benzene-insoluble fractions. The ketonic compounds in the benzene-soluble fraction were precipitated by the mercuric hydroxide-ammonia reaction, the equilin and oestrone in this precipitate being separated by the use of alcohol. By acid hydrolysis of the equilin complex in the alcoholic washings, removal of the alcohol and vacuum-distillation of the residue, a crude equilin-containing distillate was obtained from which pure equilin was crystallised by the use of 80% alcohol. In a similar way acid hydrolysis of the mercury-ketone complex yielded a residue from which the crude oestrone-containing material was concentrated by sublimation. Purification of this sublimate was accomplished by the use of the quinoline reaction (Butenandt) and subsequent crystallisation of the oestrone from alcohol.

In the case of the benzene-insoluble material the oestrone was isolated directly by use of the quinoline reaction and subsequent crystallisation from alcohol, the mercury reaction being unnecessary for this fraction. Some typical results of the amounts of oestrone (M.P. 254–256°) isolated from these benzene-insoluble fractions are given in Table IV.

Table IV. *Oestrone isolated from benzene-insoluble fraction.*

Volume unconcentrated urine l.	Colorimetric assay of "weak phenols" g.	Weight benzene- insoluble material g.	Crude oestrone (from quinoline) g.	Recrystallised oestrone g.
72	3.45	2.66	1.68	1.41
18	2.40	1.67	0.97	0.76
86	4.40	3.78	—	1.10
67	3.83	—	1.48	—
72	6.45	3.85	1.78	—

The author wishes to express his sincere gratitude to Dr G. F. Marrian for his continued interest and advice throughout the problem.

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LXXXIII. THE IONISABLE IRON IN FOODS.

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SINCE it is now generally agreed that "haematin" iron in foods is largely unavailable for haemoglobin formation, and therefore is probably not absorbed [Elvehjem *et al.*, 1933; Lintzel, 1931], it is clear that a knowledge of the total iron in a foodstuff may be of little use from a dietetic standpoint. A knowledge of the ionisable iron is probably of much more value, although it cannot be assumed that the whole of this iron will be available, or that the whole of the "haematin" iron will be unavailable. Widdowson and McCance [1936] have made use of the present figures to calculate the ionisable iron intakes of 63 normal men and the same number of women.

Ionised iron in foodstuffs and biological material may be determined by several methods [Tompsett, 1934, 1, 2; Hanzal, 1933; McFarlane, 1934; Burmester, 1934], but the use of $\alpha\alpha'$ -dipyridyl, introduced by Hill [1930], is probably the most convenient, and results obtained by means of this reagent have been found to agree in general with the biological assays of available iron in the same materials. Discrepancies have been encountered with egg yolk, but these have been satisfactorily explained [Elvehjem *et al.*, 1933; Sherman *et al.*, 1934, 1, 2].

Determinations of the ionisable iron in a number of English foodstuffs have accordingly been made, using $\alpha\alpha'$ -dipyridyl. This reagent was prepared according to the method of Hill and sodium hydrosulphite used as the reducing agent. The latter as purchased, was heavily contaminated with iron and was purified according to the directions of Hill.

Method for the determination of ionisable iron.

(a) *In flesh foods.* The raw or cooked material was cut into small pieces with a stainless steel knife and thoroughly pulped in a mortar. Five aliquots (1.5 g.) were weighed into five tubes A, B, C, D and E of 40 ml. capacity, graduated at 20 ml. and to all 10 ml. of sodium acetate-acetic acid buffer p_H 5.5 were added. Previously cooked foods were not heated, but raw foods were heated at this stage for 10 min. at 100°. After testing the p_H of the fluids in the tubes, 0.5–1.0 g. of hydrosulphite was added to all the tubes. 0.05 mg. of iron was added to tubes C and D and a few crystals of $\alpha\alpha'$ -dipyridyl to A, B, C and D. E was retained as a blank. The contents of all tubes were well mixed with a glass rod and allowed to stand overnight. 5 ml. of absolute ethyl alcohol were then added, the contents again well mixed and allowed to stand for at least 8 hours, but generally overnight. The solutions were then made up to 20 ml. with distilled water and filtered through Whatman filter-papers No. 541.

The amount of iron present in each solution was then determined by matching the colour against a series of standards in a Cole and Onslow comparator, the blank being placed in the appropriate position. A few of the extracts could have been matched in a colorimeter, but usually the natural pigments were not completely decolorised by the hydrosulphite, so that a comparator method was employed.

(b) *In fruits and vegetables.* The material was prepared for analysis as described under (a) and our portions were weighed out into the graduated tubes. 10 or 15 ml. of buffer p_H 5.5 were added and the tubes heated in a water-bath for 10 min. at 100°. After cooling, the p_H was checked, 0.05 mg. of iron added to two tubes and hydrosulphite to all. The volumes were then made up to

the graduation mark (20 ml.) with distilled water and the contents of the tubes well mixed and allowed to stand overnight. In the morning the contents were well stirred and filtered after an hour. Each filtrate was then divided into two portions. To one, a few crystals of $\alpha\alpha'$ -dipyridyl were added and the colour developed; the other served as a blank.

Standards. The iron standards were prepared as follows. In twelve tubes of uniform bore aliquots of a standard iron solution were placed corresponding to 0.0025, 0.005, 0.01, 0.02, ... 0.10 mg. of iron respectively. 10 ml. of buffer p_H 5.5 and 0.5–1.0 g. of hydrosulphite were added, followed 12 hours later by 5 ml. of absolute ethyl alcohol. Each solution was then made up to 20 ml. and the tube sealed. When the method was modified to avoid the adsorption of the coloured complex on solid vegetable material, a fresh set of standards was prepared which contained no alcohol. The absence of the latter did not influence the intensity of the colour.

Discussion of the method.

(a) *Experiments with haemin.* The use of $\alpha\alpha'$ -dipyridyl as a reagent for ionisable iron is entirely dependent upon the fact that no coloured complex is formed in the presence of haematin iron. In a personal communication, Hill informed us that free iron is liberated by the action of dissolved oxygen when hydrosulphite is added to a solution of haemin. This important point has been investigated under the experimental conditions used for the determination of ionisable iron.

4 mg. of haemin¹ were dissolved in about 3.5 ml. of $N/10$ NaOH and made up to 10 ml. 10 ml. of buffer p_H 5.5 were measured into five tubes, *A*, *B*, *C*, *D* and *E*, graduated at 20 ml. To *A* and *B* 1 ml. of the haemin solution was added and to *C* and *D* about 0.5–1 g. of hydrosulphite. After standing 15 min. (to allow all the dissolved oxygen in *C* and *D* to react with the hydrosulphite), hydrosulphite was added to *A* and *B* and 1 ml. of the haemin solution added to *C* and *D*. *E* was reserved as a reagent blank. The contents of all tubes were then thoroughly mixed, made up to the 20 ml. mark and left overnight. The solutions were then filtered, divided into two parts, one of which served as a blank; a few crystals of $\alpha\alpha'$ -dipyridyl were added to the other.

No difference could be detected between *A*, *B* and *C*, *D*, and therefore no evidence was obtained that a decomposition of haemin took place when hydrosulphite was added to a colloidal suspension of haemin at p_H 5.5 by the interaction of hydrosulphite and dissolved oxygen. There was however a very small difference between the solutions containing haemin and the reagent blank, certainly less than the 0.0025 mg. standard. It was thought that this slight difference might have been due to the presence of free iron in the original haemin solution. This was tested in the following way: 3 ml. of the haemin solution (0.4 mg. per ml.), 10 ml. of buffer p_H 5.5, and 7 ml. of distilled water were placed in a tube and thoroughly mixed. About 0.25 g. of A.R. sodium chloride was then added, the whole again well mixed and allowed to stand overnight. The solution was then filtered, which removed the haemin, and a few crystals of $\alpha\alpha'$ -dipyridyl were added. No difference could be detected between this solution and a blank prepared in a similar manner from which the haemin was omitted. Hydrosulphite, therefore, may interact with haemin and set free traces of ionised iron, but the extent to which this occurs is so very slight that it may be discounted entirely as an interfering factor in the present work.

Further, ionisable iron determinations were carried out on representative samples of the various types of foodstuffs, to which 1 ml. of the stock haemin solution was added. The results are shown in Table I. In 6 instances there was a very small and negligible increase, in 2 there was no change and in 1 a decrease.

¹ Kindly presented by R. Hill.

Table I. *Determination of ionisable iron in presence of haemin.*

Sample	Aliquot g.	Haemin added (as a solution in NaOH) mg.	Fe standard used mg.	Difference mg.
Watercress, fresh	2	None 0.4	0.03 0.0325	+ 0.0025
Bread, wholemeal	2	None 0.4	0.0325 0.0350	+ 0.0025
Apples, raw	5	None 0.4	0.0065 0.0075	+ 0.0010
Barcelona nuts	2	None 0.4	0.0625 0.0638	+ 0.0013
Turnip, raw	5	None 0.4	0.013 0.013	None
Liver, ox, raw	2	None 0.4	0.06 0.06	None
Oatmeal	1	None 0.4	0.04 0.0425	+ 0.0025
Raisins	2	None 0.4	0.04 0.0385	0.0015
Cabbage, raw	5	None 0.4	0.0275 0.0285	+ 0.0010

(b) *Recoveries.* With two exceptions (see under (h)) satisfactory recoveries of added iron were obtained from all the foods given in Tables A and B.

(c) *The accuracy obtainable with the comparator.* With most samples the final solution was perfectly clear, and it was possible to estimate the iron content of the solution to the nearest 0.0025 mg. It was always possible, even when the unknown was matched against the strong standards, to read to the nearest 0.005 mg. Solutions which gave a cloudy solution, even after centrifuging and filtering, could be matched against the standards to the nearest 0.005 mg. The percentage error was of course dependent on the above factors and on the iron content of the sample.

(d) *Incomplete extraction of the iron.* This was considered to be a possible cause of inaccuracy, and in consequence all samples were thoroughly ground in a mortar to facilitate the extraction. Experiment repeatedly showed that under the working conditions described, complete equilibrium between the tissue and the surrounding liquid had been reached when the extraction had proceeded overnight, and at least this time was always allowed.

(e) *Adsorption of the coloured complex.* Hill stated that a 30% concentration of ethyl alcohol in an acid medium prevented the adsorption of the coloured complex, but recovery estimations have always been carried out as an additional safeguard. In this way it was discovered that alcohol will not prevent adsorption of the coloured complex taking place with some vegetables and fruits. In the modification already described, the $\alpha\alpha'$ -dipyridyl was not added until the liquid had been filtered from the insoluble vegetable tissue. In this way the coloured iron complex was formed in the absence of solid material. The success of the modification can be seen from Table II, in which results obtained by the two methods are compared. It should be noted that a low recovery by the original method was associated with a low value for the ionisable iron.

It is important to add the hydrosulphite at the commencement of the extraction, since Tompsett [1934, 2] has shown that ferric iron forms complexes with

Table A. *Total and ionisable iron in foods of vegetable origin.*

Foodstuff	Systematic name	Total iron (mg./100g.)	Ionisable iron (as % of total iron)	
Vegetables:				
Artichoke, globe, cooked	<i>Cynara scolymus</i>	0.55	100	
Beans, broad, raw	<i>Vicia faba</i>	1.08	93	
butter, cooked	Var. of <i>Phaseolus vulgaris</i>	1.82	71	
French, raw	<i>Phaseolus vulgaris</i>	0.57	87	
haricot, raw	Var. of <i>Phaseolus vulgaris</i>	6.65	83	
haricot, cooked	Var. of <i>Phaseolus vulgaris</i>	2.72	84	
scarlet runner, cooked	<i>Phaseolus multiflorus</i>	0.74	74	
tinned, baked	Var. of <i>Phaseolus vulgaris</i>	2.05	98	
Beetroot, cooked	<i>Beta vulgaris</i>	0.50	94	
Brussel sprout, raw	<i>Brassica oleracea, bullata gemmifera</i>	0.67	75	
Cabbage, raw	<i>Brassica oleracea, capitata</i>	0.98	62	82
cooked	<i>Brassica oleracea capitata</i>	0.76	70	92
Carrageen moss	<i>Chondrus crispus</i>	8.88	97	
Carrot, raw	<i>Daucus carota</i>	0.56	100	
cooked	<i>Daucus carota</i>	0.41	98	
Cauliflower, raw	<i>Brassica oleracea, botrytis cauliflora</i>	0.91	99	
cooked	<i>Brassica oleracea, botrytis cauliflora</i>	0.52	100	
Celeriac, cooked	<i>Apium graveolens rapaceum</i>	0.87	98	
Celery, raw	<i>Apium graveolens</i>	0.14	100	
Chicory, raw	<i>Chichorium intybus</i>	0.57	66	59
Cucumber, raw	<i>Cucumis sativus</i>	0.25	100	
Egg plant, raw	<i>Solanum melongena, var. esculentum</i>	0.32	53	50
Endive, raw	<i>Chichorium endivia</i>	2.77	72	
Fennel, dry, raw	<i>Foeniculum vulgare</i>	9.06	45	
fresh, raw	<i>Foeniculum vulgare</i>	4.20	50	62
Horseradish, raw	<i>Cochlearia armoracea</i>	1.60	100	
Leek, raw	<i>Allium porrum</i>	0.77	91	
Lentil, raw	<i>Lens culinata, Erum Lens</i>	7.63	60	72
Lettuce, raw	<i>Lactuca sativa</i>	0.80	63	
Marrow, raw	<i>Cucurbita pepo</i>	0.44	85	
Mushroom, raw	<i>Psalidium campestris</i>	0.65	100	98
Mustard, raw	<i>Brassica</i>	1.80	50	
Mustard and cress, raw	<i>Brassica and Lepidium species</i>	5.70	45	39
Onion, raw	<i>Allium cepa</i>	0.40	100	
Parsley, raw	<i>Petroselinum sativum</i>	10.00	50	
Parsnip, raw	<i>Pastinaca sativa</i>	0.78	89	100
cooked	<i>Pastinaca sativa</i>	0.48	100	
Pea, blue, cooked	Var. of <i>Pisum sativum</i>	1.55	77	
fresh, raw	<i>Pisum sativum</i>	1.77	74	
split, raw	Var. of <i>Pisum sativum</i>	5.41	81	
split, cooked	Var. of <i>Pisum sativum</i>	1.84	71	
tinned	Var. of <i>Pisum sativum</i>	1.87	100	
tinned (Chivers)	Var. of <i>Pisum sativum</i>	2.10	100	
Potato, raw	<i>Solanum tuberosum</i>	0.60	95	100
cooked	<i>Solanum tuberosum</i>	0.62	93	100
Radish, raw	<i>Raphanus caudatus</i> or <i>R. sativus</i>	1.68	68	55
Rhubarb, raw	<i>Rheum rhabonticum</i>	0.38	100	
Salsify, raw	<i>Tragopogon porrifolius</i>	1.33	98	
Seakale, cooked	<i>Crambe maritima</i>	0.66	91	
Spinach, raw	<i>Spinacia oleracea</i>	2.96	68	
cooked	<i>Spinacia oleracea</i>	4.15	57	
Swede, raw	<i>Brassica species</i>	0.41	100	91
Turnips, raw	<i>Brassica rapa</i>	0.33	67	66
Watercress, raw	<i>Nasturtium officinale</i>	2.08	66	
Fruits:				
Apple, eating, raw	<i>Pyrus malus</i>	0.23	81	100
cooking, raw	<i>Pyrus malus</i>	0.24	100	
Apricot, dry, raw	<i>Prunus armeniaca</i>	4.08	98	
fresh, raw	<i>Prunus armeniaca</i>	0.37	95	
Avocado pear, raw	<i>Persea americana</i>	0.53	100	
Banana, raw	<i>Musa paradisiaca, subspecies sapientum</i>	0.47	100	

Table A (cont.).

		Table A (cont.).		Ionisable iron	
Foodstuff	Systematic name	Total iron (mg./100 g.)	(as % of total iron)		
Fruits (cont.):					
Blackberry, raw	<i>Rubus fruticosus</i>	0.95	41	39	
Cherry, raw	<i>Prunus cerasus</i> or <i>P. avium</i>	0.48	100		
Cranberry, raw	<i>Vaccinium oxycoccos</i>	0.70	61	79	
Currant, dry, raw	<i>Vinus vinifera</i>	1.07	89		
black, raw	<i>Ribes nigrum</i>	0.95	100		
red, raw	<i>Ribes rubrum</i>	0.66	85		
Custard apple, raw	<i>Annona squamosa</i>	0.52	100		
Damson, raw	<i>Prunus</i> species	0.63	70		
Date, dry	<i>Phoenix dactylifera</i>	1.71	82		
Fig, dry, raw	<i>Ficus aurea</i>	4.17	96		
fresh, raw	<i>Ficus aurea</i>	0.42	95		
Gooseberry, raw	<i>Ribes grossularia</i>	0.58	40		
Grape, black raw	<i>Vinus vinifera</i> species	0.27	85		
white raw	<i>Vinus vinifera</i> species	0.21	86		
dried (raisins)	<i>Vinus vinifera</i> species	3.80	97	94	
sultana	<i>Vinus vinifera</i> species	3.60	65		
Grapefruit, raw	<i>Citrus paradisi</i>	0.11	92		
Greengage, raw	<i>Prunus</i> species	0.46	84		
Lemon juice	<i>Citrus medica</i> , var. <i>Limonum</i>	0.07	80		
Loganberry, raw	<i>Rubus loganobaccus</i>	1.00	76		
Medlar, raw	<i>Pyrus germanica</i>	0.77	65		
Mulberry, raw	<i>Morus nigra</i>	1.57	50		
Nectarine, raw	<i>Prunus</i> species	0.46	87		
Olive, bottled	<i>Olea europaea</i> , var. <i>amora</i>	1.03	97		
Orange juice	<i>Citrus aurantium</i>	0.20	85		
Passion fruit, raw	<i>Passiflora</i>	1.12	100		
Peach, dry, raw	<i>Prunus persica</i>	7.60	92		
fresh, raw	<i>Prunus persica</i>	0.30	100		
Pear, cooking, raw	<i>Pyrus communis</i>	0.16	94		
dessert, raw	Var. of <i>Pyrus communis</i>	0.21	100	100	100
Pineapple, raw	<i>Ananas sativus</i>	0.22	91		
Plum, cooking, raw	<i>Prunus communis</i>	0.35	60	50	
dried (prunes), raw	<i>Prunus communis</i>	3.20	72		
Victoria, raw	<i>Prunus communis</i>	0.30	47	60	
Pomegranate juice, raw	<i>Punica granatum</i>	0.20	33	33	
Quince, raw	<i>Cydonia vulgaris</i>	0.19	54		
Raspberry, raw	<i>Rubus adocens</i>	1.11	82	69	
Strawberry, raw	<i>Fragaria vesca</i>	0.71	52		
Tangerine juice, raw	Var. of <i>Citrus aurantium</i>	0.18	95		
Tomato, raw	<i>Solanum lycopersicum</i>	0.37	73	53	66
Nuts:					
Almond	<i>Prunus amygdalus</i>	4.54	99		
Barcelona	<i>Corylus maxima</i> , <i>barcelonensis</i>	3.44	91		
Brazil	<i>Bertholletia excelsa</i>	2.70	61	62	
Chestnut, baked	<i>Castanea vulgaris</i>	0.87	60	42	
Cob	<i>Corylus maxima</i> or <i>C. avellana</i>	1.44	94	100	
Cocoonut	<i>Cocos nucifera</i>	1.98	86		
Cocoonut milk	<i>Cocos nucifera</i>	0.10	75		
Peanut	<i>Arachis hypogoea</i>	1.19	100		
Walnut	<i>Juglans regia</i>	1.83	41		
Cereal and Cereal foods:					
Biscuit, Digestive	--	1.57	91		
Biscuit, Ryvita	--	3.20	100		
Bread, brown, Daren		2.96	100		
Goldmedal	--	2.30	100		
Hovis	-	2.48	95		
Lyons (whole-meal)	--	2.12	72	76	
wholemeal	.	3.43	83		
Bread, white, Lyons	--	1.07	87	90	
Flour, white	<i>Triticum sativum</i>	1.00	93		
Oatmeal	<i>Avena sativa</i>	4.15	96		
Rice	<i>Oryza sativa</i>	0.45	85		

Table A (cont.).

Foodstuff	Systematic name	Total iron (mg./100 g.)	Ionisable iron (as % of total iron)
Miscellaneous:			
Beer, Bitter. Watneys	—	0.04	100
Burton. Watneys	—	0.06	100
Cocoa	—	14.20	93
Chocolate, Milk	—	1.67	84
Plain	—	3.28	89
Treacle, Golden syrup	—	1.68	95
Black	—	9.17	100

Table B. Total and ionisable iron in flesh foods.

Foodstuff	Systematic name	Total iron (mg./100 g.)	Ionisable iron (as % of total iron)
Fish:			
Cod, raw	<i>Gadus morrhua</i>	0.34	100
Haddock, steamed	<i>Gadus aeglefinus</i>	0.75	100
Halibut, raw	<i>Hippoglossus vulgaris</i>	0.44	100
Herring, fried	<i>Clupea harengus</i>	1.02	74
(Bloater) fried	—	1.29	31
raw	—	1.02	39
(Kipper) boiled	—	1.38	58
Mackerel, fried	<i>Scomber scombrus</i>	1.17	64
Plaice, steamed	<i>Pleuronectes platessa</i>	0.68	97
Prawns, boiled	<i>Leander serratus</i>	0.62	81
Rock salmon (catfish), raw	<i>Anarrhichus lupus</i>	0.36	44
Roe (herring), raw	—	0.63	99
Salmon, "Shipmate" tinned	—	0.89	94
Sardines "Skipper" tinned	—	3.44	65
Skate, raw	<i>Raja batia</i>	0.33	100
Sole, steamed	<i>Pleuronectes microcephalus</i>	0.45	100
Winkles, boiled	<i>Littorina littorea</i>	10.4	58
Muscular organs. Animal and Bird:			
Beef, raw	—	3.54	11
roast	—	5.20	22
corned, tinned	—	3.34	35
Chicken, white flesh, roast	—	1.60	31
red flesh, roast	—	2.70	24
Heart, baked	—	5.83	63
Mutton, roast	—	5.10	24
Pig products, Bacon, raw	—	2.50	29
Chop, fried	—	1.40	47
Ham, cooked	—	4.45	15
Rabbit, stewed	—	1.89	42
Tongue, cooked	—	5.80	19
Veal, raw	—	0.93	45
roast	—	1.35	55
Glandular organs:			
Kidney, Ox, stewed	—	4.02	66
Pig, fried	—	9.50	58
Liver, Calf's, raw	—	13.30	100
Lamb's, fried	—	2.76	100
Ox, raw	—	6.70	78
Pig's, raw	—	20.00	80
Sweetbreads, raw	—	1.47	71
Miscellaneous:			
Egg, raw	—	2.50	100
Sausage, Beef, fried	—	4.18	72
Pork, fried	—	3.39	81

Table II. *Comparison of methods for ionisable iron.*

Nature of material	Weight taken for analysis g.	Original method			Modified method		
		Ionisable iron in sample mg.	Iron added to sample mg.	Iron recovered mg.	Ionisable iron in sample mg.	Iron added to sample mg.	Iron recovered mg.
Currants, Black	5	0.025	0.05	0.01	0.05	0.05	0.05
" Red	5	0.012	0.05	0.033	0.028	0.05	0.049
" Dried	2	0.012	0.05	0.025	0.019	0.05	0.051
Loganberries	5	0.017	0.05	0.035	0.038	0.05	0.05
Raspberries	5	0.025	0.05	0.043	0.035	0.05	0.05
Cocoa	1	0.016	0.05	Traces only	0.135	0.05	0.05

certain proteins and will not react with $\alpha\alpha'$ -dipyridyl. Hydrosulphite reduces the iron, and the ferrous iron, which does not form these complexes, will then react with the dipyridyl.

(f) The p_H was always tested before the hydrosulphite was added, and if not between p_H 5.4 and 5.8 was brought within these limits by the necessary additions. Sherman *et al.* [1934, 2] have reported that they have experienced some difficulty in this respect with animal tissues, but we have only had to reinforce the buffer in the case of some acid fruits.

(g) *Impurities.* The hydrosulphite has already been discussed. Ordinary filter-papers were found to contain iron, sometimes in large amounts, but Whatman papers No. 541 were reasonably satisfactory. Reagent blanks were carried out from time to time, filtered and placed in the comparator when matching unknowns. These blanks were generally less coloured than the 0.0025 mg. standard.

(h) *Interference with colour development.* As judged by recoveries this only occurred twice. Blackberries were found to contain a substance which prevented ferrous iron from reacting with $\alpha\alpha'$ -dipyridyl. The natural pigment of the fruit was completely decolorised by the hydrosulphite, so that no difficulty was experienced in matching the solutions. The filtered juice behaved in a similar manner, but the interference observed was variable in amount. The figure given in Table A for the available iron in this fruit was obtained from a sample which gave very slight inhibition of colour development, but the result may still be too low. It was also impossible to obtain satisfactory recoveries from walnuts, and the figure given for the ionisable iron is probably too low.

Determination of total iron.

Samples were incinerated according to the method of McCance and Shipp [1933]. Such foodstuffs as liver, raw sweetbreads and fruits with a high sugar content tend to ash badly, and in these cases the ash was moistened with a little water, two or three drops of concentrated hydrochloric acid were added, the whole was placed on a hot plate until dry, and the ash was then again incinerated. When only traces of carbon were left, the ash was cooled, moistened with water and heated with 2.5 ml. of concentrated a.r. HCl. 10 ml. of approximately $N/2$ HCl were added, the contents of the crucible brought to the boil and filtered into a 100 ml. graduated flask. Three further extractions of the small charcoal residue (if any) were made using 10 ml. of $N/2$ acid each time. The filter-paper was washed with boiling water until the contents of the flask were near the graduation mark. When cool, the volume was made up to 100 ml. with distilled water. The iron in an aliquot of this solution was determined by means of thioacetic acid [Lyons, 1927]. The presence of pyrophosphate does not interfere with the development of the colour. The incineration and determination were always carried out in duplicate. $\alpha\alpha'$ -Dipyridyl was also used to determine the total iron in some foodstuffs by a method somewhat similar to that used by Hill and Ketlin [1933]. In general the results obtained by both methods were in close agreement,

but with meat $\alpha\alpha'$ -dipyridyl tended to give low results. Since the determination of total iron by $\alpha\alpha'$ -dipyridyl was originally undertaken only as a further test of the value and scope of this reagent, the iron figures obtained by incineration have been preferred in all cases of difference and are the only ones given in this paper. The low results obtained by the $\alpha\alpha'$ -dipyridyl method are due to the incomplete decomposition of haemin compounds.

Sources of materials.

Material to be analysed was purchased from the Amalgamated Fruiterers, the Army and Navy Stores and local shops. Many of the figures were obtained on mixed samples from all three sources, but this has not always been possible and some determinations have been made on material purchased at one shop only. This applies particularly to rare fruits and to estimations of common materials which have been carried out for confirmatory purposes. The figures given for meat and fish apply in most instances to samples from one source.

RESULTS.

Tables A and B contain the figures which have been obtained for total and ionisable iron. The figure for total iron is the mean of the results found on the separate occasions on which the material was analysed. The ionisable iron is given as a percentage of the total iron, and the results for different occasions are given separately. The materials analysed have been classified under vegetables, fruits, nuts, fish and meats, and are arranged in alphabetical order within each group.

Discussion of the results.

These figures for ionisable iron, depending as they do upon two determinations with independent errors, cannot be claimed to have a high degree of accuracy, even though each determination was carried out in duplicate and with the greatest care. When cloudy solutions were obtained, which made matching in the comparator difficult, an error of 10–15 % was possible, but this is not incompatible with valuable results for dietetic purposes. Percentages of the same order have been obtained when individual members of a group of similar foodstuffs have been estimated, or when the same foodstuff has been analysed more than once. There are many instances of this in Table A. The results for the cereal group, the dried and fresh apricots, the figs, and the plums and prunes illustrate this. The results for the herring family and the gadoid group of demersal fish are equally good examples. This is the more interesting because the total iron may vary considerably from one occasion to another, and it would seem from the results that the percentage of the total iron in ionisable form is perhaps a more characteristic feature of any particular material than the total amount of iron in it. This is illustrated by the figures shown in Table III. It will be seen that gross fluctuations in the total iron are not accompanied by variations in the percentage of that iron which reacts with $\alpha\alpha'$ -dipyridyl. It is for this reason that the figures for total iron in Tables A and B have been averaged. This communication is not concerned with the variability of the total iron in food materials, or with the best average figure for dietary purposes. The figures in Table A will be found in some instances to be the same as those given for total iron by McCance *et al.* [1936].

(1) It would seem therefore that the ionisable iron of groups of foodstuffs, as determined by these methods and expressed as a percentage of the total, may be accepted with considerable confidence. Individual variations from the group

Table III. *Constancy of ionisable iron as percentage of the total iron.*

Foodstuff	1		2	
	Total iron (mg./100 g. wet weight)	Ionisable iron (as % of total iron)	Total iron (mg./100 g. wet weight)	Ionisable iron (as % of total iron)
Cauliflower	0.91	99	0.52	100
Cranberry	1.11	61	0.25	79
Egg plant	0.32	50	0.27	53
Fennel	9.06	45	4.10	49
Kidney	4.92	66	9.50	58
Mushroom	0.95	100	0.35	98
Mustard and cress	1.80	50	4.70	45
Pomegranate juice	0.15	33	0.24	33
Potato	0.85	95	0.40	100
Raisin	3.80	97	5.40	94
Tomato	0.47	66	0.20	74

figure have always been confirmed at least once before being included in Table A, so that they also may be accepted as having significance.

(2) It will be observed that a number of values for available iron are stated in Table III as being 100 % of the total. If cytochrome is a constituent of every aerobic cell this cannot be strictly true. It is certain also that some blood must have been included with liver and white fish, so that in these cases also a figure of 100 % should be accepted with reserve. The reason for obtaining figures of 100 % may be inaccuracies inherent in the method, the destruction of cytochrome by cooking, or by the heating for 10 min. at 100° to which raw materials were subjected. It is, however, probable from the experiments with haemin and the small percentage of ionisable iron found in meat, that under our experimental conditions negligible quantities of haematin are decomposed. Destruction by heating does not matter, for most foods are cooked before being eaten, so that for dietetic purposes the figures may be accepted.

(3) There are very few previous figures with which to compare the present ones. Elvehjem *et al.* [1933] reported that 47 % of the iron in wheat was "available" and 57 % of that in oats. The present results are higher, but our experience has been that the method used by Elvehjem may give, with plant products, results which are far too low (Table II). Rose *et al.* [1934; Vahlteich *et al.*, 1935] moreover, have found that wheat and bran are excellent sources of available iron. Sherman *et al.* [1934, 2] found that more than 60 % of the iron in liver was available and 50 % of that in beef muscle. According to the present findings the former of these seems rather low and the latter definitely too high. The figure obtained by these authors for soya bean agrees with the present group figure for legumes, but the present figure for spinach is higher than their figure obtained with $\alpha\alpha'$ -dipyridyl and agrees with their figure for available iron determined by an acid extraction method.

(4) Considering the present series of results it has been found that: (a) At least 75 % of the iron in cereals reacts with $\alpha\alpha'$ -dipyridyl. The average is much higher and may be taken to be about 90 %. (b) Only some 10–25 % of the iron in beef and mutton so reacts. Other flesh foods contain a variable but higher percentage of their iron in reactive form. Almost the whole of the iron in liver seems to be in ionisable form. (c) All the iron in most white fish reacts with $\alpha\alpha'$ -dipyridyl, but there are exceptions, *e.g.* rock salmon (catfish). Herrings, mackerel and sardines contain only about 60 % of their iron in inorganic form. (d) The iron in most nuts is mainly ionisable but there are exceptions, *e.g.* Brazil nuts. (e) Green-leaf vegetables contain 60–70 % of their iron in inorganic form.

Cauliflower and globe artichokes, which are generally classed with the green vegetables but which are not really green leaves, contain nothing but inorganic iron. (f) Root and stem vegetables give variable results. Some contain only inorganic iron. Potatoes contain 90–100 % of their iron in this form. (g) Fresh and dried legumes contain 70–80 % of their iron as inorganic iron. Tinned beans and peas were found to contain all their iron in reactive form and this difference may be due to destruction of the organic compounds during the preserving. It is perhaps worth noting that tinned meat also contained a much higher percentage of its iron in the inorganic state than fresh or domestically cooked meat. Compare also the results for cocoa, which have been confirmed on several occasions, with the average figure for the legumes. (h) 50–100 % of the iron in most fruits is ionisable, and percentages of the same order have generally been found within each species. In plums, prunes and green gages, for example, about 75 % of the iron was found to be in ionisable form. In cherries, apricots and peaches, however, nearly 100 % of the iron was ionisable. 80–100 % of the iron in apples and pears was found to be ionisable. Tomatoes contained only 50–70 % of their iron in this form.

SUMMARY.

1. The general applicability of $\alpha\alpha'$ -dipyridyl to the determination of ionisable iron in foodstuffs has been investigated, and methods have been described for its use with plant and animal materials.
2. The ionisable iron in 155 British foodstuffs has been determined by means of $\alpha\alpha'$ -dipyridyl. Total iron has also been determined in these foods.
3. The percentage of the total iron in ionisable form may be a more characteristic feature of any particular material than the total amount of iron in it.

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LXXXIV. THE FATTY ACIDS OF OX BLOOD.

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WHILST numerous data concerning the nature of the fatty acids present in depot fat and liver are available [Banks and Hilditch, 1931; Klenk and Schoenebeck, 1932; Irving and Smith, 1935], there are few observations on the nature of the fatty acids in blood. This problem is clearly of importance, since knowledge as to the transport of fatty acids from the tissues and depots *via* the blood can only be obtained when the chemical nature and relative proportions of the various fatty acids present in the blood of animals in the post-absorptive state are known. As a contribution to this problem the present work was undertaken.

EXPERIMENTAL.

The lack of knowledge on this subject is due to the great difficulty of preparing fatty acids from blood in sufficient quantity to make adequate analysis possible. Whilst it might be anticipated that such a preparation would be readily achieved by digestion of the blood with alkali, followed by acidification and extraction with a fat solvent, such as ether, great difficulties are encountered. In the first place the amount of total fatty acid present in different forms of combination in blood is relatively small (about 0.2 g. per 100 ml.), while a further difficulty arises because of the high protein concentration in blood. Repeated experiments showed that simple digestion with alkali, followed by acidification, resulted in the precipitation of such a considerable quantity of protein degradation products that extraction with any fat solvent was most ineffective and it happened quite often that the proportion of fatty acid extracted in this way was an insignificant part of the whole. Endeavours were made to overcome these difficulties by hydrolysis of the blood with 10% NaOH in the presence of alcohol. Some fifty varieties of treatment with different proportions of 10% NaOH and alcohol and variations in time of heating were investigated, but in no case was the result satisfactory. Many experiments of similar type were carried out in which the strength of alkali was varied from 15 to 30%, with heating for different time periods in the presence of varying proportions of alcohol. The method finally adopted, which was the best compromise possible in view of the large volume of blood required, was as follows. To each 100 ml. of blood were added 5 g. NaOH and the mixture was refluxed for 4 hours on a sand-bath; after cooling overnight, 50 ml. of alcohol were added and the mixture was refluxed for 2 hours more; it was then cooled, acidified with conc. HCl and the fatty acids and unsaponifiable matter extracted with ether. After washing the ethereal extract with water and evaporating to dryness, the residue was saponified with *N* sodium ethoxide and the fatty acids and unsaponifiable matter prepared in the usual manner and purified by treatment with light petroleum. As a control of this and the other methods investigated, further samples were treated by the method of Channon and Collinson [1929] and the yields compared. It appeared that the method yielded about 70% of the total fatty acids present in blood. A

large scale preparation was then carried out in which 51.7 l. of blood were treated in volumes of 3-4 l. at a time to give 78 g. of fatty acids (151 mg./100 ml.). The corresponding figures quoted from a large number of previous samples of ox blood by Channon and Collinson averaged 180 mg./100 ml. By combining the fatty acids obtained from these various trial experiments with the main bulk, the total fatty acids available weighed 96 g., i.v. 92.6; those obtained by the latter authors had i.v. 97.5.

Separation of saturated and unsaturated acids. 95.4 g. of the mixed acids were fractionated by the Twitchell process as modified by Hilditch and Priestman [1931]. Considerable difficulty was experienced in obtaining a more saturated fraction which had a reasonably low i.v., but ultimately the acids were divided into two portions: (1) 25.6 % of the mixture, i.v. 9.6, and (2) 72.4 %, i.v. 121.6, mol. wt. 302, the loss being 2.0 %. In order to determine the amount of saturated acid still remaining in the liquid fraction, Bertram's oxidation process was applied, when 3.777 g. of that fraction yielded 0.274 g. acids, (i.v. 4.6) or 7.3 %. Assuming that oleic acid was the cause of the i.v. of 4.6 and 9.6 quoted above, the true amounts of saturated and of unsaturated acids in the original mixture were calculated to be 27.9 and 70.1 % respectively, excluding the loss of 2.0 %.

Analysis of the saturated fraction. 20.7 g. acids (i.v. 9.6) were esterified and the methyl esters distilled into five fractions and a residue. The acids from each fraction were separated from traces of unsaponifiable matter and their mol. wt. and i.v. determined. The composition of each was then calculated, the results being shown in Table I.

Table I. *Fractionation of the saturated esters.*

Fraction	B.P. at 3 mm. up to ° C.	Wt. of ester g.	i.v. of acids	Mol. wt. of acids	Composition %			
					Palmitic	Stearic	Ligno- ceric*	Oleic
1	160	1.48	3.2	257	6.7	—	—	0.2
2	168	5.49	3.4	263	19.3	5.3	—	1.0
3	173	3.03	4.4	272	6.0	7.4	—	0.7
4	181	3.63	6.0	275	5.6	10.2	—	1.1
5	185	3.00	7.5	283	0.5	12.3	—	1.2
Residue	—	4.81	15.2	321	—	8.6	10.0	3.8
Total	—	21.44	—	—	38.1	43.8	10.0	8.0

* For purposes of calculation the acids higher than stearic acid have been grouped together as lignoceric acid.

Table II. *Fractionation of the unsaturated esters.*

Fraction	B.P. at 3 mm. up to ° C.	Wt. of esters g.	Twitchell separation (description)	i.v. of acids	Mol. wt. of acids	Saturated			Unsaturated		
						C ₁₆	C ₁₈	C ₂₁	C ₁₈	C ₂₀	C ₂₂
1 a	155	1.09	Solid	53.6	277	0.3	0.2	—	0.8	—	—
b	—	—	Liquid	106.7	291	—	—	—	2.7	1.3	—
2 a	160	2.07	Solid	35.5	283	—	1.2	—	0.8	—	—
b	—	—	Liquid	110.7	293	—	—	—	4.6	3.5	—
3 a	170	2.19	Solid	32.2	290	—	0.9	0.1	0.5	—	—
b	—	—	Liquid	106.7	291	—	—	—	6.2	2.9	—
4	175	3.26	..	115.8	293	—	—	—	9.1	6.8	—
5	176	3.22	..	118.5	294	—	—	—	8.4	7.3	—
6	176	2.90	..	130.0	309	—	—	—	—	14.1	—
Residue	—	4.87	..	152.2	325	—	—	—	—	9.3	14.4
Total	—	*19.60	—	—	—	0.3	2.3	0.1	33.1	45.2	14.4

* 20.56 g. were taken initially. Loss = 4.7 % of the total unsaturated fractions or 3.4 % of the original mixed acids.

Analysis of the unsaturated acids by bromination. The results obtained by brominating the unsaturated acids of blood were very similar to those previously found and fully discussed in an analysis of pig liver fatty acids [Irving and Smith, 1935]. 22.3 g. (i.v. 121.6) yielded 3.37 g. ether-insoluble bromide, m.p. 228° (indef.), Br 67.9%. $C_{20}H_{32}O_2Br_8$ requires Br 67.8%. The minimum amount of arachidonic acid present was therefore 4.9% of the unsaturated acids.

From the ether-soluble portion, 1.98 g. crude tetrabromostearic acid were isolated, m.p. 106°, Br 56.1%. After prolonged fractional crystallisation, the purest sample weighed 0.35 g., m.p. 110–111°. $C_{18}H_{32}O_2Br_4$ requires m.p. 114°, Br 53.3%. Obviously this derivative was still contaminated with more complex bromides from which it could not readily be separated.

By the usual method of extracting the ether-insoluble bromides with benzene, no hexabromostearic acid was found, suggesting that linolenic acid was entirely absent or present in traces only.

Oxidation of the unsaturated acids. 12.78 g. of the liquid fraction (i.v. 121.6) were oxidised and twice reoxidised by the process of Lapworth and Mottram [1925, 1], as carried out by Channon *et al.* [1934], a method which is recognised to give the greatest yield of dihydroxy-derivatives so far obtainable from such a mixture. The dihydroxystearic acid so prepared weighed 1.51 g. Recrystallisation yielded 1.02 g., m.p. 131°, mol. wt. 314, and 0.23 g., m.p. 123°. The usual 9:10-dihydroxy-derivative requires m.p. 132°, mol. wt. 316.

1.01 g. dihydroxystearic acid were now further oxidised by the method of Lapworth and Mottram [1925, 2]. The resulting dibasic acid (0.341 g.) melted after recrystallisation at 141°, mol. wt. 177. $C_8H_{14}O_4$ requires m.p. 141°, mol. wt. 174 and the theoretical weight obtained should have been 0.556 g. It can be said therefore that the bulk of the octadecenoic acid in ox blood is of the usual $\Delta^9,^{10}$ type.

The tetrahydroxystearic acid arising from the original oxidation of the 12.78 g. amounted to 0.36 g. and was the usual mixture of isomerides melting indefinitely at 155°.

The amounts of di- and tetra-hydroxy-derivatives obtained correspond to 10.5 and 2.3% of oleic and linoleic acids in the unsaturated fraction, but it is well known that with such a mixture these values are always very considerably below the theoretical figures. As shown in Table III, attempts were made to

Table III. *The oxidation of the acids from each liquid fraction from the distillation of the unsaturated esters.*

Fraction	Wt. taken for oxidation g.	Dihydroxystearic acid			Tetrahydroxystearic acid		
		Weight g.	m.p. ° C.	Oleic acid in total unsaturated acids %	Weight g.	m.p. ° C.	Linoleic acid in total unsaturated acids %
1 b	0.433	0.035	126	0.3	0.004		0.04
2 b	1.064	0.077	125	0.5	0.098	160–163	0.6
3 b	1.330	0.225	126	1.4	0.129	160–163	0.7
4	2.029	0.351	127	2.5	0.109	160	0.7
5	2.074	0.388	127	2.6	0.213	164–165	1.3
6	2.014	0.144	126	0.9	0.034	163	0.2
Residue	3.467	None	—	—	None	—	—
Total	—	—	—	8.2	—	—	3.5

increase these yields in order to obtain more accurate values by oxidising the different fractions which resulted on distillation of the liquid esters. However, no appreciable difference was observed [*cf.* Irving and Smith, 1935].

Analysis of the unsaturated acids by fractional distillation followed by oxidation of each fraction. 21.48 g. acids (i.v. 121.6, mol. wt. 302) were esterified and the methyl esters distilled. The fractions were saponified, traces of unsaponifiable material removed and the acids prepared. The total loss in the whole process amounted to 4.7 % of the unsaturated fraction and, since much decomposition had been observed to take place in the residual fraction during the distillation with the formation of petroleum-insoluble products, it may be assumed that the loss was mainly in that residual fraction. Since the acids of the first three fractions were partly solid at room temperature, they were subjected to the Twitchell separation.

From the mol. wt. and i.v. recorded in Table II, it was possible to calculate the mean mol. wt. of each liquid fraction, had it been fully saturated. Then from these calculated figures the proportions of C_{18} , C_{20} and C_{22} acids were estimated. From the data available, only the mean degree of unsaturation can be known for each of these types of acid.

A portion of each fraction was now oxidised by Lapworth and Mottram's method, the object being to obtain as true a value as possible for the amounts of oleic and linoleic acids present in the total mixture. The results are set out in Table III.

The figures obtained in this way for the contents of oleic and linoleic acids in the unsaturated fraction were therefore 8.2 and 3.5 %, a result which is discussed later.

Hydrogenation of the liquid fraction. As a liquid saturated acid has been isolated by Turner [1931] from cat's kidney, it was desirable to investigate the possibility of the existence of such substances in blood. Such acids, if present, would be found in the liquid fraction from the Twitchell process. 4.41 g. mixed acids from a separate preparation were therefore separated by the lead soap process and 1.3 g. of the liquid fraction hydrogenated. The hydrogenated acids, which were recovered quantitatively, formed a hard solid crystalline mass, m.p. 58–60°. The absence of more than traces of liquid saturated acids was therefore established.

DISCUSSION.

The value of the general analysis just described depends in the first place on the truth of the assumption that the particular preparation of the fatty acids employed was truly representative of the whole. In the present case the amount of fatty acids extracted from the blood was approximately 70 % of the figure obtainable on a small scale by the method of Shimidzu [1910], and 85 % of that obtained by the method of Chaumon and Collinson [1929]. As there is no apparent reason why in the method used here any particular acid should be isolated in preference to another, it seems probable that the mixture prepared was truly representative.

It must also be observed that samples of fatty acids prepared from different small batches of ox blood vary quite appreciably both in mean i.v. and in mean mol. wt., but the results recorded in this communication may claim to be typical of the average, since the analysis was made on the mixed acids from over 50 l. of blood.

The fractional distillation method of making the general analysis has already been discussed by Hilditch [1934] and by Irving and Smith [1935]. In regard to the results in Table I, where the content of palmitic and stearic acids is calculated as a percentage of the saturated fraction and in Table II, where the C_{18} and C_{20} unsaturated acids are presented as percentages of the unsaturated

fraction, it is realised that the figures quoted for these particular acids may only approach the true values to within ± 5 units %. This possible error is considerably reduced, particularly in the case of the saturated acids, when the composition of the whole mixture is finally calculated as recorded in Table IV.

Table IV. *A comparison of the fatty acid content of the liver, blood and depots.*

		Ox depot* fat (Average of 4 analyses)	Ox blood fat	Ox liver fat†		Pig liver‡ fat
				Phosphatide	Glyceride	
Saturated acids	Lower acids	6	—§	Trace	Trace	1
	C ₁₆	27	10	12.5	25	14
	C ₁₈	22	13	27	20	19
	Higher acids	Trace	3	Trace	Trace	2
	Total	55	26	39.5	45	36
Unsaturated acids	C ₁₆	—	§	5	9	1.5
	C ₁₈	45	26	27	37	33
	C ₂₀	—	33	18	8	20
	C ₂₂	—	10	10.5	1	7.5
	Total	45	69	60.5	55	62

* Banks and Hilditch [1931].

† Klenk and Schoenebeck [1932].

‡ Irving and Smith [1935].

§ There was not sufficient material available to make possible the detection of small amounts of lower acids.

In the case of the higher saturated acids the total amount present was of the order of 3 %. It made no significant difference to this figure whether the actual acid was regarded as arachidic or lignoceric. In actual fact there are probably traces of several higher saturated acids in ox blood, but lignoceric acid was chosen for calculation purposes, as it is known to be present in sphingomyelin blood.

Hydrogenation experiments proved the absence of anything more than minute traces of liquid saturated acids, while the absence of ordinary linolenic acid was indicated by bromination. These findings are in agreement with the corresponding results obtained in the case of pig liver fat [Irving and Smith, 1935].

Attempts were made to estimate the amounts of oleic and linoleic acids present in the liquid fraction, first by oxidising a sample of the total mixture and then by oxidation of each individual fraction obtained on distillation. The figures of 10.5 and 2.3 % resulting in the first case for the contents of oleic and linoleic acids were confirmed, within the limits of the processes involved, by those of 8.2 and 3.5 % by the second method. Obviously nothing was gained by the latter and much more laborious process. A very similar finding resulted in the case of the pig liver acids where the corresponding pairs of figures were 21.4 and 4.5 % and 22.0 and 4.3 % respectively. Again nothing was gained by the more elaborate method. It must also be noticed that in the two cases under discussion the amount of C₁₈ unsaturated acids found to be present by fractional distillation was very considerably more than that suggested by the oxidation results. The poor yields of hydroxy-derivative always obtained from such mixtures remains unexplained.

The composition for the total mixture of blood fatty acids is shown in Table IV where it is compared with the figures found for ox depot fat by Banks and Hilditch [1931] and with those obtained for ox liver fat by Klenk and

Schoenebeck [1932]. The pig liver values are also quoted for comparison. From the i.v. recorded in Table II, together with the oxidation results already quoted, it appears probable that the 26 % of C_{18} acids present in the total mixture consisted approximately of 20 % oleic and 6 % linoleic acid. From the i.v. and bromination results the C_{20} and C_{22} unsaturated acids contained anything from one to four or five double bonds.

One of the most interesting points arising from this table is the very high content of C_{20} and C_{22} unsaturated acids occurring both in blood and in liver. Apart from the possibility of minute traces, these substances are conspicuously absent both from the depot fat and from various foodstuffs, such as grass [Smith and Chibmall, 1932]. It is thus possible that these acids are synthesised in the tissues, or alternatively that their presence is explained by the fact that they are contained in the foodstuffs in traces too small for detection and that these small amounts are selectively retained in the tissues. From the relatively large amounts shown in Table IV it might be tentatively supposed that they are the result of synthesis, although further confirmation is desirable before a definite conclusion is drawn.

Another fact of interest arising from the figures in Table IV, together with the i.v. and bromination experiments already recorded here and in other papers cited, is the absence of linolenic and the relatively small proportions of linoleic acid present in the blood, liver and depots, whereas grass and other vegetable foodstuffs are known to contain very appreciable quantities of these substances. This finding confirms the view that the more unsaturated acids of the diet are those which are most readily and quickly oxidised by the body and may well be connected with the fact that these particular acids are "essential" in the diet [Burr and Burr, 1929, 1930; Burr *et al.*, 1932].

As there are no measurable amounts of higher unsaturated acids in the depots and yet the liver and blood contain them to such an extent, it might be suggested that the liver has perhaps synthesised these higher constituents and that the blood is transporting them to tissues other than the depots. Results in partial confirmation of such a theory have arisen from work being carried out in these laboratories on the total fatty acids prepared by hydrolysis of the carcass of the rat from which the liver and the alimentary tract have been removed. Some 10 % of these total acids have been shown to consist of C_{20} and C_{22} unsaturated compounds, although in the depots alone they are almost entirely lacking [Banks *et al.*, 1933].

SUMMARY.

A method of preparing the fatty acids from blood on a large scale was investigated and the acids from over 50 l. of ox blood were obtained in yields approximating to 70 % of the estimated content. These were analysed and the final results are set out in Table IV. The presence of the higher unsaturated acids in liver and blood is discussed.

The authors wish to express their indebtedness to Prof. H. J. Channon for his valuable advice and encouragement during the course of this work.

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LXXXV. THE PRODUCTION OF FREE SULPHUR FROM L-CYSTINE BY A SOIL BACTERIUM.

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IN the ordinary putrefaction of protein material the protein-sulphur is liberated mainly as hydrogen sulphide accompanied in a few cases by relatively small amounts of mercaptans. The present paper is an account of an organism which is capable of the aerobic degradation of L-cystine with the direct liberation of free sulphur.

In earlier work on the bacterial decomposition of cystine the medium used has always contained other sources of carbon such as carbohydrates or peptone, owing apparently to the difficulty of obtaining an organism capable of developing on cystine alone. Thus Sasaki and Otsaki [1912], who found that nineteen out of twenty-one different pure cultures formed hydrogen sulphide from cystine, employed Fränkel's medium containing asparagine, ammonium lactate and inorganic salts. Bürger [1914] also used this medium or a cystine medium which contained meat extract and lead acetate. He examined twenty-three different species of bacteria and observed hydrogen sulphide production in every case but never detected mercaptans.

The question of mercaptan formation from cystine is still not settled: Kondo [1923] in a review of the available evidence concludes that the mercaptan arises from the interaction of hydrogen sulphide with substances derived from carbohydrates or histidine, and that in the absence of these the sulphur is liberated as hydrogen sulphide.

During the course of our work a somewhat different mode of attack was described by Tarr [1933] who, using washed cells of *Proteus vulgaris*, quantitatively investigated the decomposition of cystine under anaerobic conditions. In this case too the sulphur appeared almost quantitatively as hydrogen sulphide, the cystine molecule yielding two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.

In order to maintain the conditions of our own experiments as simple as possible an attempt was made to find some organism capable of utilising cystine as the sole source of sulphur, nitrogen and carbon. From garden soil a bacterium was isolated which was found to be able to attack cystine somewhat slowly in the absence of all other compounds of carbon, nitrogen or sulphur. On examination of the products it was found that free sulphur and free ammonia were produced in approximately equimolecular proportions and this sulphur appears to arise from the cystine without the intermediate formation of detectable amounts of hydrogen sulphide.

EXPERIMENTAL.

Isolation of the bacterium.

A modified Czapek medium containing cystine was inoculated with a suspension of soil obtained from the grounds surrounding the laboratory buildings.

¹ Open Science Research Scholar, University College, Nottingham, 1932-34.

The medium had the composition: KNO_3 , 2.0 g.; K_2HPO_4 , 1.0 g.; MgCl_2 , $6\text{H}_2\text{O}$, 0.5 g.; KCl , 0.5 g.; FeCl_3 , 0.01 g.; water to 1 l. Several test-tubes each containing 10 ml. of the solution and approximately 0.1 g. cystine were inoculated with a few drops of the soil suspension. After three or four days one tube showed signs of bacterial activity. A loopful of this active liquid was transferred into a sterile tube of the medium and after a week from this into a third tube of the sterile cystine medium. On subsequent plating out on to nutrient agar the culture was found to be homogeneous and was transferred from the plates on to ordinary nutrient agar slopes.

Characteristics of the organism.

Morphology. Short bacillus. $2.0-2.5\mu$ by 0.5μ occurring singly, in pairs or in short chains.

Motile. The usual flagella stains and mordants failed however to reveal flagella at any stage of the cultivation.

Gram-negative.

Gelatin liquefied. Gelatin stab, napiform.

Agar colonies. Round; after 3 days at 20° diameter 0.75 mm.; after 7 days 2-3 mm.; after 40 days 5-6 mm.

Colonies, amorphous, homogeneous, homochromous. Surface smooth. Edges entire. Colour, grey-white by reflected, brown by transmitted light. Opalescent. Elevation between pulvinate and capitate.

Agar slant. Flat, dirty white with faint suggestion of a yellowish central streak.

Growth on agar completely inhibited at 37° .

Non-sporing. Suspensions in sterile water sterile after 20 min. at 80° .

Litmus milk. Alkaline, not coagulated.

Carbohydrate-peptone media. Lactose, sucrose, fructose, galactose, xylose and glucose, growth visible in one day, no gas, alkali production.

Potato slope. Smooth, raised, slightly yellowish orange streak becoming gradually browner.

Nitrate-peptone. Nitrate reduced to nitrite. Ammonia formed in traces only.

Gelatin shake. Small circular colonies on free surface, slowly eating into medium and producing complete liquefaction from above downwards.

Indole formed in 1% peptone solution.

Origin. Soil.

A culture has been deposited at the National Collection of Type Cultures.

On the Bergey system of nomenclature [Bergey, 1934] the organism would appear to be a species of the genus *Achromobacter* and differs in several characteristics from any described hitherto.

In consideration of the characteristic utilisation of cystine the name *Achromobacter cystinovorum* is suggested.

Method of experiment.

For the actual experiments the medium used for the isolation of the organism was further simplified by the elimination of sulphate, nitrate and magnesium, preliminary trials having shown that growth was not retarded by the absence of any or all of these. The medium finally employed consisted of K_2HPO_4 , 1.28 g.; KCl , 0.5 g.; hydrated FeCl_3 , 0.01 g.; water, 1 l., to which weighed amounts of cystine were added into each flask just prior to sterilisation. The weight of cystine usually employed was 0.5 g. per 100 ml. of the medium; this amount dissolved at 100° during sterilisation and partly crystallised out on cooling. The p_{H} of this medium was 7.0.

It will be seen that the medium contained no source of carbon, sulphur or nitrogen other than cystine.

The cystine used was prepared from human hair, was decolorised several times by charcoal in boiling hydrochloric acid solution, filtered, precipitated from the filtrate by sodium acetate and washed with water. A further purification was effected by treatment with warm ammonia solution (1 vol. sp. gr. 0.880 to 1 vol. water) filtering in order to remove traces of earthy phosphates, and precipitating the cystine from the filtrate by the addition of acetic acid. Finally the product was exhaustively extracted with hot water in order to remove residual tyrosine.

(Found S (Denis-Benedict method) and N (Kjeldahl): Sample 1: S, 27.1, 27.1; N, 11.4, 11.6%. Sample 2: S, 26.25, 26.7; N, 11.7, 11.6%. Cystine requires S, 26.7, N, 11.7 %.)

1.0052 g. cystine in 100 ml. of 1.004 N hydrochloric acid gave $[\alpha]_{D}^{20} - 260.2^{\circ}$ and $[\alpha]_{D}^{20} - 222^{\circ}$.

For preliminary qualitative work 11. flat-bottomed round flasks, each containing 500 ml. of the medium were used. Signs of activity usually appeared on the third day after inoculation and incubation at 20°. The medium became turbid and later the powdery cystine deposit gave place to a colourless sludge-like mass. On shaking the contents of the flask with ether and evaporating the washed ethereal extract crystals of sulphur were obtained. For the quantitative determination of the free sulphur, the total contents of the culture flask were acidified with hydrochloric acid and filtered through a weighed Gooch crucible, and the residue was washed and dried at 100° to constant weight. The crucible and contents were together extracted with carbon disulphide and again weighed, and the sulphur was calculated as the difference between the weights. This figure was in several cases checked by collecting the carbon disulphide extract in a weighed beaker and weighing the residue of pure crystalline sulphur. The sulphur values were also checked against uninoculated control flasks.

The absence of hydrogen sulphide and indeed of sulphydryl compounds generally was confirmed by frequent withdrawal of samples of the active medium and testing with ammonium sulphide and sodium nitroprusside. Sulphates too were entirely absent at every stage. Nitrogen determinations by Kjeldahl's method were carried out on several complete culture flasks at various stages of growth, and in every case, even where the superincumbent air had been continuously removed for carbon dioxide estimation, there was no loss of total nitrogen.

The ammonia production was determined either by formaldehyde titration of filtered samples periodically removed from the culture flask or by adding excess of sodium carbonate to the total culture and aspirating air through the liquid at 40° into standard acid. Separate control experiments showed that cystine itself gave no ammonia under these conditions.

Carbon dioxide formation was measured by aspirating a current of air over the surface of the culture into standard barium hydroxide solutions.

Estimation of sulphur and ammonia. 250 ml. Erlenmeyer flasks were used, each containing 100 ml. of the cystine medium. After the sulphur had been removed by filtration through a weighed Gooch crucible as previously described an aliquot part of the total acid filtrate was made alkaline by addition of sodium carbonate and the ammonia determined by aspiration into standard acid.

Allowing 0.001 g. for S and 0.002 g. for N in the controls the atomic ratios for S/ammonia-N shown in the right-hand column of Table I are obtained.

Simultaneous estimation of carbon dioxide, sulphur and ammonia. A train of bottles was set up in the following order. Potassium hydroxide solution 30%, dilute barium hydroxide solution, dilute sulphuric acid, the culture flask, dilute sulphuric acid, two Drechsel bottles each containing

Table I.

Wt. of cystine in 100 ml. medium, g.	Time from first sterili- sation days	Time after inoculation days	S produced g.	Ammonia-N produced g.	S/NH ₃ -N
Controls. Flasks merely subjected to discontinuous sterilisation.					
0.5000	6		0.0004	---	—
0.5000	14	—	0.0024	0.0018	—
0.5000	20	—	0.0013	0.0020	—
0.5006	7	—	0.0004	0.0006	---
0.4999	15	—	0.0004	0.0013	---
0.5000	21	—	0.0001	0.0016	—
0.4999	42	—	0.0008	0.0017	—
Inoculated flasks					
0.4999	13	7	0.0536	0.0248	1.01
0.4997	20	14	0.0830	0.0361	1.05
0.5008	27	21	0.0650	0.0321	0.94
				0.0313	
0.5009	15	8	0.0483	0.0219	0.95
0.4998	21	14	—	0.0294	—
0.4998	42	35	0.0801	0.0284	1.26
				0.0305	

measured volumes of standard barium hydroxide solution with phenolphthalein and finally a soda-lime tube connected to the aspirator. The air current was passed over the surface of the liquid culture medium by means of two tubes, a short inlet tube which passed just through the rubber stopper, and a longer outlet tube which reached almost to the surface of the medium. The evolved carbon dioxide was estimated by back-titration of the baryta bottles with standard barium hydroxide solution if they showed excess of carbon dioxide, or otherwise by allowing the barium carbonate to settle, pipetting off an aliquot part of the clear pink solution and titrating with standard HCl.

At the end of each experiment the sulphuric acid bottle was examined for ammonia, but as had been found in all previous experiments, none was present. Before disconnecting the culture flask its contents were cautiously acidified with hydrochloric acid and a rapid stream of air passed through the apparatus. The contents of the flask were then filtered through a tared Gooch crucible and sulphur and ammonia estimated in residue and filtrate respectively. After extraction of the residue with carbon disulphide for the removal of sulphur, the residue was in some cases examined for total nitrogen, *i.e.* synthesised bacterial protein-nitrogen. (See Table II.)

Table II.

Exp. no.	Cystine in medium g.	Time after inoculation days	Sulphur produced mg.	Ammonia-N produced mg.	CO ₂ produced mg.	Atomic ratio S/ammonia-N/CO ₂ -C
1	0.5010	17	50.8	20.6	123	1.1 : 1 : 1.9
2	0.5003	9	31.8	16.8	88	0.83 : 1 : 1.66
3	0.5005	13	37.6	14.9	127	1.11 : 1 : 2.73
4	0.4995	12	41.4	17.1	97	1.06 : 1 : 1.43
5	0.4996	8	11.6	10.3	92	0.49 : 1 : 2.9
6	0.5004	7	11.7	5.8	51	0.88 : 1 : 2.9
7	0.4998	7	11.2	6.5	66	0.76 : 1 : 3.3

In the last two experiments the S/N/CO₂ ratio was further checked (*a*) by a determination of bacterial protein-nitrogen in the solid residue after extraction with carbon disulphide and (*b*) by a determination of non-ammonia-nitrogen in the acid filtrate (Table III).

LXXXVII. ACTION OF ESTERASE IN THE PRESENCE OF ORGANIC SOLVENTS.

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METHODS.

IN the method of studying esterases elaborated by the author, the active preparations are placed in contact with non-aqueous solutions of the substrates. The organic solvents used were either soluble, such as acetone, or practically insoluble in water, such as benzene, carbon tetrachloride and others.

The active esterase preparations used in the majority of the experiments were obtained from pig pancreas.

Prep. A (tissue pulp) was obtained by passing the fat-free pancreas three times through a mincing machine and triturating the product in a mortar.

The dry prep. B was obtained by shaking tissue pulp for 1 hour with five volumes of acetone, decanting the supernatant liquid and repeating the process. After squeezing out the solvent the preparation was spread on filter-paper and air-dried. The dry preparation was then ground in a coffee mill; the final product contained 8-12% of water.

The activity of this preparation depends in great measure on the water content. When this is low, an approximate proportionality may be found between the water content and the velocity of reactions catalysed by the preparation [Sym, 1933, 1].

In the case of prep. C the aqueous extract of the esterase was obtained by shaking the dry preparation described above with 5 volumes of water for 10 min., centrifuging and passing the centrifugate repeatedly through a layer of infusorial earth. The activity of the clear extracts obtained was studied after adding 10 g. of sodium butyrate or acetate to 100 ml. of extract.

Prep. D was obtained by evaporating prep. C (without sodium salt) in flasks at 20 mm. pressure until the residue had the consistency of a compact gel adherent to the bottom of the flask. The gelatinous mass so obtained is more resistant to inactivating factors than the extract from which it originated and it may be used repeatedly.

The above preparations exhibit esterification activity only in the presence of water. Thus preps. A, B and D are reversibly inactivated by further dehydration, being reactivated by addition of water.

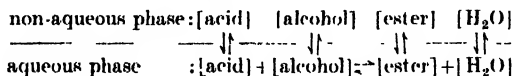
Systems containing organic solvents insoluble in water, such as benzene, can, as in the case of prep. C, be of varied water content without the activity being destroyed. But when water-soluble organic solvents are used, the enzyme undergoes irreversible inactivation in presence of higher concentrations of water [Sym, 1933, 1].

The action of the enzymic preparations was studied in most cases in systems containing *n*-butyl alcohol (*M* BuOH), 0.43 *M* butyric acid and benzene.

The velocity of the reactions was studied by determining the initial velocity of esterification, *v*, *viz.* the number of millimols. of ester per litre which is formed in the non-aqueous phase during the first hour of reaction.

5 ml. of the benzene layer were titrated with 0.1 *N* alcoholic NaOH using *o*-cresolphthalein as indicator. The reactions were conducted in a Köhler water thermostat with constant shaking and mostly carried out with two samples. Determinations of acidity were made before the reaction started with 5 ml. of each of the two samples. After some time a second determination was made for one of the samples and some time later one for the second sample.

The following considerations illustrate the mechanism of reactions in systems of the type described. After addition of the benzene solution to the aqueous enzyme preparation the substrates dissolved in the benzene diffuse into the aqueous phase where they enter into reaction under the influence of the enzyme. The resultant products are then partitioned between the aqueous and the non-aqueous phases. With the conclusion of the reaction, two equilibria are established: an equilibrium of reaction in the aqueous phase and an equilibrium of distribution of the substrates and of the products of reaction between the aqueous and the non-aqueous phases. These equilibria may be represented as follows:



The application of organic solvents practically insoluble in water gives much higher yields of esters (often over 95 %) than the use of aqueous systems owing to the fact that the products of reaction are removed from the medium where the reaction proceeds. The reaction of hydrolysis of esters can be investigated by using water-soluble organic solvents with a certain limited content of water (such as acetone, pyridine *etc.*).

In view of the fact that the volume of non-aqueous phase taken was large in comparison with that of the aqueous phase and that the partition coefficients of the substrates and reaction products were such as to favour greater concentration in the non-aqueous phase, the latter may be regarded as a reservoir of the components of the reaction supplying substrates to the aqueous phase and removing esters from it. The progress of the reaction is conveniently studied in the non-aqueous phase without the necessity of removing enzyme from the system.

Preliminary experiments.

The velocity of the reaction rises rapidly with increasing comminution of the pancreas tissue, whilst for prep. B the differences in the size of the grains do not influence the value of *v*. In shaken systems of preps. A and C, the reaction proceeds about twice as quickly as in unshaken systems. Shaking and the magnitude of interfaces have no influence on the velocity of systems in which prep. D is present.

The results of experiments on the effect of varying the relative amounts of preps. A, B, and C with and without shaking are given in Table I.

The initial velocity of reaction was in all cases found to be proportional to the quantity of prep. B taken whilst in the case of prep. A this proportionality was observed only in systems containing 1 g. and less of preparation.

In the case of prep. C, *v* is proportional to the volume of preparation taken for shaken systems containing 10 ml. of extract but not for unshaken ones.

Finally the effect of varying the amount of prep. D was examined by evaporating 7 ml. of extract C in one flask and 14 ml. in another, to yield films of about 35 sq. cm. in area: 12.5 ml. of 2 *M* BuOH and 0.43 *M* butyric acid in benzene were added to each flask. The values of *v* found were 7.9 and 19.1 millimols. per litre per hour, respectively (at 37.1°), whence it follows that the

Table I. *Influence of varying the relative amounts of esterase on the velocity of reaction.*

The systems contain 20 ml. of 0.43 *M* butyric acid and *M* BuOH in benzene. Temperature 37°. The systems were shaken.

Amount of preparation g.	<i>v</i>			
	With pulp (A)		With dry prep. (B)	
	In system	Per g.	In system	Per g.
0.25	16.2	64.8	15.2	60.8
0.5	29.3	58.6	36.4	72.8
1.0	62.6	62.6	77.7	77.7
2.0	89.9	44.9	149.5	74.8
4.0	116.0	29.0	—	—

The systems contain 50 ml. of benzene solution of 0.43 *M* butyric acid, *M* BuOH, and 2.5–20 ml. of extract C. Temperature 37°.

Vol. of extract taken ml.	<i>v</i>			
	Unshaken systems		Shaken systems	
	In system	Per ml. of extract	In system	Per ml. of extract
2.5	2.9	11.6	3.9	15.6
5.0	5.2	10.4	8.1	16.2
10.0	5.9	5.9	15.2	15.2
20.0	7.8	3.9	23.3	11.7

velocity of reaction was greatly dependent on the thickness of the films. In another case, in which both substrates were practically insoluble in water, 75 ml. of 0.4 *M* oleic acid and 0.3 *M* cetyl alcohol in benzene were added to the films prepared from 15 and 30 ml. of prep. C; the values of *v* found were 1.9 and 2.9 m.mols. per litre per hour.

It must be accepted that all substrates, whether readily or difficultly soluble in water, react in the aqueous phase. It is possible that the aqueous phase contains some substance which enhances the solubility of practically insoluble substrates such as cetyl alcohol and oleic acid; it will be shown in a future paper that the presence of bile acids and soap, which form soluble complexes with such substrates, greatly accelerates reaction in systems containing two liquid layers.

The author expressed the opinion in a previous paper [1930] that the reaction between oleic acid and glycerol took place at the phase-boundary, at which a pellicle of esterase formed. This opinion should now be modified: the reaction takes place in the thickness of the pellicle formed.

It has now been found that reversible inactivation commences when the water content in preps. A and D falls below 25 % and is well marked in preparations of the type of A, C and D, dried in a high vacuum at 50° to a water content of 5 %. This supports the view that the reaction takes place in the water of imbibition of the esterase; dehydration beyond a certain point leads to breaks in the continuity of this medium, thereby rendering impossible diffusion of substrates to, and of reaction products from, the enzymic surface.

The activity of 2 g. of prep. B in a system consisting of 25 ml. of *M* BuOH and 0.43 *M* butyric acid in CCl₄ at –20° was found to be quite considerable (about 50 % of ester after 24 hours); a second identical system to which water had been added was inactivated in these conditions. The conclusion might be drawn that the water of imbibition remains liquid at –20°, whilst in the second system, containing free water, a layer of ice forms in which diffusion cannot take place.

The addition of salts such as sodium acetate, butyrate, oleate or carbonate and aqueous ammonia prevents excessive acidification and hence greatly accelerates reaction in the presence of preps. A, B and D, and is a condition of the activity of prep. C. The results of experiments illustrating these points are given in Table II.

Table II. *Accelerating action of Na acetate and butyrate.*

Systems contain 20 ml. of *M* BuOH and 0.43 *M* butyric acid in benzene, 1.5 g. of prep. A, or 0.5 g. of prep. B and Na acetate or butyrate. Acceleration is expressed as % activation as compared with systems without salts.

Amount of salt added g.	% activation			
	By Na acetate		By Na butyrate	
	Prep. A	Prep. B	Prep. A	Prep. B
0.05	—	34	—	46
0.1	—	35	—	60
0.15	50	—	111	—
0.2	—	25	—	43
0.3	44	—	96	—
0.4	—	6	—	5
0.6	47	—	Inactivation	—
0.8	—	—	—	Inactivation
1.2	6	—	Inactivation	—
2.4	Inactivation	—	—	—

Influence of concentration of substrates on the velocity of enzymatic esterification.

It has been shown [Sym, 1931; 1933, 1, 2] that in solvents soluble in water such as acetone the value of the velocity coefficient rises continuously with increase in concentration of one or both of the substrates (except in those cases in which increase in concentration of the acid leads to inactivation of the enzyme). In the case of solvents not soluble in water (benzene, CCl_4) progressive increase in the concentration of alcohols leads to a maximum value of v at a certain concentration, above which the value of v falls; increasing the concentration of acid, on the other hand, leads to a continuous increase in v .

The results (Table III) show that v is roughly doubled by raising the concentration of acid from 0.215 to 0.43 *M*; increasing the concentration to 0.86 *M*

Table III. *Influence of varying the concentration of butyric acid on the velocity of esterification with BuOH.*

Systems contain 20 ml. of *M* BuOH and 0.215–1.72 *M* butyric acid in benzene, with preps. A, B, C, and Na acetate. The values are given in brackets in those cases in which gradual inactivation of the enzyme took place.

Initial conc. of butyric acid <i>M</i>	1 g. of prep. A with 0.07 g. of Na acetate		1 g. of prep. B with 0.1 g. of Na acetate		5 ml. of prep. C with 0.5 g. of Na acetate	
	Concn. of ester after 48 hours		Concn. of ester after 48 hours		Concn. of ester after 72 hours	
	v m.mol.	m.mol.	v m.mol.	m.mol.	v m.mol.	m.mol.
0.215	72	210	390	208	6.6	188
0.43	112	361	686	408	11.9	386
0.86	118	684	695	794	17.0	(378)
1.72	Rapid inactivation	—	(330)	(919)	Rapid inactivation	—

does not to any great extent further increase the value of v , whilst concentrations of 1.72 M in all cases inactivated the enzyme.

The effect of varying the concentration of alcohol is given in Table IV, from which it appears that maximum values of v are obtained for preps. A, B, C and

Table IV. *Influence of varying the concentration of BuOH on the velocity of esterification with butyric acid.*

Systems contain 20 ml. of 0.25-8 M BuOH and 0.43 M butyric acid in benzene, with preps. A, B, C and D. Temperature 37°.

Initial concn. of BuOH <i>M</i>	2.5 g. of prep. A		1 g. of prep. B		5 ml. of prep. C with 0.5 g. of Na butyrate		Prep. D from 20 ml. of prep. C	
	Concn. of ester after 45 hours		Concn. of ester after 45 hours		Concn. of ester after 120 hours		Concn. of ester after 216 hours	
	<i>r</i> m.mol.	m.mol./litre	<i>r</i> m.mol.	m.mol./litre	<i>v</i> m.mol.	m.mol./litre	<i>v</i> m.mol.	m.mol./litre
0.25	44	181	132	220	1.9	189	9.9	250
0.5	67	322	202	380	3.3	345	14.4	404
1.0	138	382	240	412	4.7	386	23.2	414
2.0	143	372	200	412	5.2	386	19.4	402
4.0	129	366	129	386	3.6	351	16.2	402
8.0	79	259	109	367	2.0	215	—	—

D in presence of 1-2 M BuOH. This phenomenon is, as will be seen later, partly ascribable to variations in the partition coefficients of the substrates between the aqueous and non-aqueous phases due to changes in the concentration of BuOH.

Velocity of esterification of different alcohols and acids, varying the concentration of alcohols.

The results given in Table V indicate that for different alcohols maximum values for v are obtained when molar concentrations are taken and that in general, with the exception of isopropyl alcohol, the velocity of esterification falls with increase in the molecular weight of the alcohol.

Table V. *Effect of varying the concentration of different alcohols on the velocity of esterification with butyric acid.*

Systems contain 20 ml. of 0.43 M butyric acid and 0.125-4 M alcohol in benzene or 20 ml. of 0.43 M butyric acid in alcohol, and 1 g. of prep. B. Temperature 37°. Final concentration of ester determined after 7 days.

Initial concn. of alcohol M	<i>n</i> -Propyl		<i>iso</i> Propyl		<i>n</i> -Butyl		<i>iso</i> Amyl		<i>n</i> -Octyl		Cetyl alcohol	
	r m.mol.	Concn. of ester m.mol./litre	r m.mol.	Concn. of ester m.mol./litre	r m.mol.	Concn. of ester m.mol./litre	r m.mol.	Concn. of ester m.mol./litre	r m.mol.	Concn. of ester m.mol./litre	r m.mol.	Concn. of ester m.mol./litre
0.125	146	—	2.7	114	58	—	76	120	55	123	19	120
0.25	204	—	4.6	209	90	230	86	240	76	240	31	240
0.5	284	384	5.9	301	141	390	99	406	92	394	51	408
1.0	316	398	8.2	373	154	410	126	416	104	416	57	410
2.0	180	396	7.0	327	135	402	101	396	59	400	52	382
4.0	152	—	6.2	262	79	398	7.3	394	—	—	—	—
Alcohol taken as solvent	Inactivation	—	—	—	35	394	44	390	14	388	92(?)	396

The effect of varying the concentration of BuOH was examined for a number of acids. The results given in Table VI indicate that in benzene the velocity of reaction falls with increasing molecular weight of the acids and that the highest values of v are obtained also with molar concentrations with the exception of stearic acid.

Table VI. *Effect of varying concentration of BuOH on the velocity of esterification with different acids.*

Systems contain 20 ml. of 0.43 M acid and 0.125–4 M BuOH in benzene or 20 ml. of 0.43 M acid in BuOH and 1 g. of prep. B. Temperature 37°.

Concn. of BuOH M	<i>n</i> -Butyric		<i>n</i> -Hexanoic		Lauric		Stearic acid	
	Concn. of ester after 168 hours		Concn. of ester after 168 hours		Concn. of ester after 168 hours		Concn. of ester after 168 hours	
	v m.mol. litre	m.mol. litre	v m.mol. litre	m.mol. litre	v m.mol. litre	m.mol. litre	v m.mol. litre	m.mol. litre
0.125	32	124	29	123				
0.25	50	248	41	210	15	223	5	232
0.5	75	398	53	380	26	293	9	292
1.0	82	412	62	390	40	400	13	398
2.0	70		48	384	40	392	16	390
4.0	44	394	40	—	34	380	19	392
BuOH as solvent	15	394	31	354	28	350	22	406

Optimum conditions of determining the esterase activity of various preparations.

On the basis of the above experiments, the following standard procedure is recommended for various preparations. The pig pancreas is treated as for prep. A above. 1 g. portions of pulp are added to 25 ml. of 0.5–0.1 M BuOH and 0.43 M butyric acid in benzene, and v is determined after 30 and 60 min.

In the case of other tissues (liver, stomach, intestines), 5 g. of pulp should be taken, the concentration of butyric acid should not exceed 0.1–0.2 M , and that of BuOH should be 1.0 M in 25 ml. benzene solution. Addition of 0.5 g. of Na acetate is necessary for these tissues. The systems should be very vigorously shaken, and the second titration should be made after 12–24 hours at 37°.

When preparations of the type of prep. B of pancreas are taken, the systems should consist of 0.5 g. of preparation with 25 ml. of benzene solution as for prep. A. The second titration should be performed after 30–60 min.

Aqueous or 20 % glycerol extracts of prep. B should always contain 10 % of sodium acetate or butyrate. The systems should consist of 5 ml. of extract and 25 ml. of benzene solution as for prep. A. The second titration should be made after 5–6 hours.

Films of the type of prep. D are obtained from 10 ml. of aqueous extract. 12.5 ml. of benzene solution as for prep. A are added, 2.5 ml. being taken for titration; the second titration is performed after 3–4 hours.

When it is desired to compare the activities of preparations of the type B care should be taken that the water content of these preparations is not too low.

Partition of substrates between water and benzene taken in various proportions.

The partition of butyric acid and BuOH between benzene and water in presence of different concentrations of alcohols was determined with the object of elucidating the influence of the concentration of the substrate on v . It is

realised that the solvent power of water for butyric acid and BuOH in systems containing enzyme preparations may differ from that of pure water, but the difference is probably not great.

The results, given in Table VII, indicate that the concentration of acid in the aqueous phase in all cases falls with increasing concentration of alcohol.

Table VII. *Partition coefficients of butyric acid between benzene and water in presence of different concentrations of alcohols.*

Systems consist of 50 ml. of 0.8-0 *M* alcohol and 0.43 *M* butyric acid as initial concentration in benzene, and 10 ml. of water. Temperature 14.5°.

Concn. of alcohol	<i>n</i> -Propyl	<i>n</i> -Butyl	<i>n</i> -Octyl	Cetyl	<i>iso</i> Propyl alcohol
0	1.80	1.80	1.80	1.80	1.80
0.25		2.23	2.30	2.38	
0.5	2.27	2.44	2.57	2.60	2.20
1.0	2.92	3.02	3.48	3.41	2.67
2.0	4.06	4.35	5.02	-	3.80
4.0	6.47	6.40			6.0
8.0	8.0	7.5			-

The values for alcohols practically insoluble in water are fairly close to each other, whilst those for lower alcohols are on the whole lower. It would appear from these results that the values of the partition coefficients are independent of the number of C atoms in the alcohol but are in some way related to solubility of the alcohol in water.

The partition coefficients at 18°, determined in systems containing 10 ml. of water and 50 ml. of benzene in which 0.215, 0.43, 0.86, and 1.72 *M* butyric acid and *M* BuOH were dissolved, were respectively 3.1, 3.1, 3.8 and 4.5 showing that the concentration of acid in the aqueous phase on the whole rises with increasing concentration in benzene. Thus the rise of *v* with the rise in the concentration of acid is explained.

The concentration of butyric acid in water is lowered by addition of sodium butyrate as was shown by determining the partition coefficient of the acid. It follows that the activating effect of sodium butyrate must be due chiefly to its influence on the acidity of the aqueous phase. Thus the p_H of the aqueous phase of the system, 50 ml. of 0.43 *M* butyric acid and *M* BuOH in benzene + 10 ml. of water, was 3.4 as compared with 5.9 when the aqueous phase contained 1 g. of sodium acetate.

The concentration of BuOH in the aqueous phase after the attainment of equilibrium between the benzene and the aqueous phase was determined in the following manner. 50 ml. of a solution of 0.43 *M* butyric acid and of BuOH in various concentrations in benzene were shaken for 15 min. with 10 ml. of water. After 24 hours 4 g. of anhydrous K_2CO_3 were dissolved in 5 ml. taken from the aqueous layer. This solution was shaken with 10 ml. of *i*-amyl ether, 5 ml. of the resulting solution were taken and 1.6 g. of CaO added. After 24 hours the content of BuOH in the dried solution of *i*-amyl ether solution was determined by means of the method of Tschugaeff and Zerewitinoff [Houben-Weyl, 1930]. For the initial concentrations of BuOH in benzene, 0.25, 0.5, 1.0 and 4.0 *M*, the following concentrations of BuOH in water were noted: 0.075, 0.18, 0.29, 0.45 and 0.55 *M* respectively (temperature: 14.5°).

On the basis of the above data on the partitions of substrates it is now possible to attempt to solve the problem whether there is some connection

between v and the concentration of the substrates in the aqueous phase. From the theoretical point of view the following equation can be expected:

$$v = k [\text{butyric acid}] [\text{BuOH}].$$

50 ml. of water were added to 15 g. of prep. C. After removing the larger particles of tissue from the mixture by centrifuging, 5 ml. of the turbid centrifugate were taken for reaction. Such an aqueous phase containing esterase in the form of suspended particles of prep. B does not need sodium acetate for the maintenance of activity. Table VIII gives the results of these experiments.

Table VIII. *Dependence between the concentration of substrates in the aqueous phase and the velocity of enzymic esterification.*

25 ml. benzene solution of 0.43 M butyric acid and BuOH in various concentrations (5 ml. of the benzene solution taken for the first titration), 5 ml. of enzyme preparation. Temperature 14.5°.

Concn. of BuOH in benzene M	Concn. of butyric acid in water M	Concn. of BuOH in water M	r m.mol.	$v[\text{acid}] [\text{BuOH}]$ $= k$
0.25	0.164	0.075	0.029	2.4
0.5	0.132	0.18	0.042	1.8
1.0	0.095	0.29	0.055	2.0
2.0	0.064	0.45	0.054	1.9
4.0	0.054	0.55	0.044	1.5

The values of k obtained indicate that the dependence of v on concentrations of BuOH in the systems studied is primarily influenced by the concentrations of substrates in the aqueous phase.

Application of the method of Tschugaeff and Zerewitinoff to the determination of enzymic esterification.

In the method described for the examination of esterase, enzymic esterification can be followed not only by determining the decrease in acidity but also by determining the drop in alcohol concentration. The method of Tschugaeff and Zerewitinoff can be applied for the latter determinations as the following experiment proves.

50 ml. of a benzene solution containing 0.5 M BuOH and 0.5 M butyric acid were subjected to esterification by means of 2 g. of prep. B. The temperature of reaction was 37°. The determination of acidity was carried out as above. The determination of the drop in alcohol concentration was effected by adding 2.5 g. of anhydrous K_2CO_3 to 5 ml. of the filtered benzene solution in order to remove the butyric acid. After shaking and centrifuging, 1 ml. was taken from the upper layer of the solution for determination of hydroxyl groups. It was found that after 1 hour the concentration of butyric acid dropped by 84 millimols./litre. The drop in the concentration of BuOH during the same time was 98 millimols./litre. After 2 hours, the drop in acid concentration was 195 millimols./litre and that in alcohol concentration 210 millimols./litre. Thus it can be seen that there are certain deviations, but the method described can undoubtedly be further improved.

SUMMARY.

1. A method for the determination of the activity of esterase preparations has been described depending on the addition to the given preparations of solutions in organic solvents of the substrates. The degree of esterification can

be determined in the solvent phase on the basis of the drop in acidity and alcohol concentration.

2. The velocity of reaction v rises with increasing concentration of acid, whilst when the concentration of alcohol is raised the velocity of reaction rises to a maximum at molar concentration of alcohol, thereafter falling.

3. This influence of concentrations of butyric acid and BuOH on v has been explained to some extent by the aid of coefficients of partition of substrates of esterification between the benzene and the aqueous phase.

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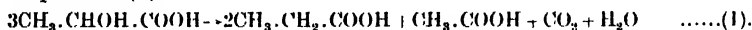
LXXXVIII. MECHANISM OF GLUCOSE DISSIMILATION BY THE PROPIONIC ACID BACTERIA.

By HARLAND GOFF WOOD AND CHESTER HAMLIN WERKMAN.

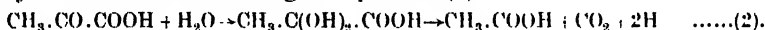
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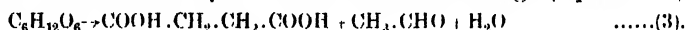
THE first work of importance on the chemistry of the propionic acid fermentation was that of Fitz [1878] who suggested that the dissimilation of lactic acid occurs according to equation (1).



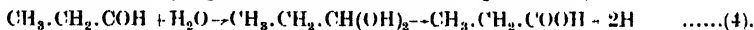
Virtanen [1923; 1925], Virtanen and Karström [1931] and Van Niel [1928] supposed that glucose after preliminary phosphorylation is converted into two molecules of the C_3 type, one being oxidised to acetic acid and CO_2 whilst two molecules are reduced to propionic acid. Van Niel suggested that pyruvic acid is an intermediary, dissimilated according to equation (2).



Virtanen [1923] first noted the formation of succinic acid in glucose fermentations and suggested that it was formed by a 4- and 2-carbon cleavage (equation 3).



A number of the proposed intermediate compounds have been isolated or detected. Foote *et al.* [1930] and Fromageot and Tatum [1933] found lactic acid, Virtanen and Karström [1931] hexosemonophosphate, Pett and Wynne [1933] methylglyoxal and Wood and Werkman [1934, 1] pyruvic acid. Wood and Werkman [1934, 2] isolated propionaldehyde and offered more definite information on the mechanism of propionic acid formation (equation 4).



They [1934, 3] detected a non-reducing material and studied the mechanism of the fermentation.

The present investigation deals with the mechanism of glucose dissimilation by several species of *Propionibacterium*. Results have been obtained which show the dissimilation to be more complex than that proposed by previous investigators.

METHODS.

The analytical procedure was similar to that described by Wood and Werkman [1936]. The sugar determinations were made before and after fermentation according to the method of Munsen and Walker [1906]. Non-reducing sugars were determined after hydrolysing with 2.25 % HCl for 2.5 hours and determining the increase in reducing sugars. The CO_2 was absorbed in soda-lime or in standard NaOH and the volatile acids and non-volatile acids were determined as previously described. Purity of the cultures was verified. Constituents of the medium were sterilised separately and mixed at the time of inoculation. The inoculum was equivalent to 5 % by volume and consisted of cultures grown in a medium containing 0.5 % glucose. Fermentations were conducted under nitrogen in litre flasks. All cultures used have been identified and described by Wood and Werkman [1936] and Werkman and Brown [1933]. Culture numbers and species

are as follows: 52W and 48W, *P. shermanii*; 49W and 36W, *P. pentosaceum*; 34W, *P. arabinosum* and 11W, *P. petersonii*. Culture 48W is culture 5 of Foote *et al.* [1930]. The results are calculated as described by Wood and Werkman [1936]. The non-reducing material is calculated as a 6-carbon sugar.

EXPERIMENTAL.

The results presented in Table II are unlike others reported in the literature and schemes suggested by previous investigators do not meet the requirements of these data. The ratios of propionic acid to acetic acid vary from 2.13 to 14.72;

Table I. *Conditions of experiments shown in Table II.*

Culture and fermentation no.	Temperature of incubation °C.	Period of incubation days	Age of inoculum days	Medium			Volume of medium fermented ml.
				Glucose %	CaCO ₃ %	Bacto-yeast extract %	
34W ₁	37	18	5	3.0	1.4	0.5	700
34W ₂	30	18	5	2.1	1.0	0.35	990
49W ₃	37	15	5	3.0	1.4	0.5	700
49W ₄	30	15	5	3.0	1.4	0.5	700
36W ₅	37	24	5	3.0	1.4	0.4	700
48W ₆	37	24	5	3.0	1.4	0.4	700
52W ₇	30	40	2	3.0	1.5	0.4	800
52W ₈	30	40	2	3.0	1.5	0.4	800
11W ₉	30	12	5	2.4	2.5	0.5	800
11W ₁₀	30	12	5	2.4	2.0	0.7	800

Table II. *Dissimilation of glucose by the propionic acid bacteria.*

Quantities produced per 100 m.mol. of fermented glucose

Culture and fermentation no.	Glucose fermented per litre m.mol.	Propionic acid m.mol.	Acetic acid m.mol.	CO ₂ m.mol.	Succinic acid m.mol.	Non-reducing material m.mol.	Ratio CO ₂ /acetic acid	Ratio propionic/acetic acid	C recovered %	Redox index
34W ₁	145.3	130.1	17.8	63.6	13.2	2.8	3.59	7.82	97.6	1.01
34W ₂	109.5	148.8	10.1	63.6	7.9	3.7	6.29	14.72	97.4	0.91
49W ₃	155.9	127.8	16.6	52.3	21.1	2.8	3.15	7.70	95.0	0.98
49W ₄	156.9	116.3	22.7	42.6	23.8	3.7	1.88	5.13	92.5	0.94
36W ₅	169.7	122.7	12.2	41.5	26.1	1.4	3.38	10.08	91.2	0.89
48W ₆	78.6	114.2	34.1	50.7	11.3	—	1.49	3.35	84.5*	0.99
52W ₇ †	164.1	104.0	44.6	47.4	12.6	4.3	1.06	2.33	88.0	1.03
52W ₈ ‡	163.9	106.1	45.9	47.0	15.0	4.1	1.02	2.33	91.2	1.02
11W ₉	133.0	114.8	54.0	51.5	11.8	8.9	0.95	2.13	100.6	1.00
11W ₁₀	133.0	117.8	55.4	51.2	8.1	—	0.92	2.13	91.3*	0.94

* Non-reducing material was not analysed. C recovered would not be complete.

† 0.8 m.mol. of lactic acid produced per 100 m.mol. of fermented glucose.

‡ 1.5 m.mol. of lactic acid produced per 100 m.mol. of fermented glucose.

the ratios of CO₂ to acetic acid from 0.92 to 6.29, whilst the succinic acid fluctuates from 7.9 m.mol. to 26.1 m.mol. per 100 m.mol. of fermented glucose. Virtanen and Van Niel found the ratios of CO₂ to acetic acid to be substantially 1. The ratio of propionic to acetic acid usually has been found to be approximately 2 although Van Niel found ratios as high as 5. This increased yield of reduced products was explained by a donation of hydrogen by compounds occurring in the yeast-water medium. Such an explanation is not acceptable in the present case inasmuch as the redox indexes show that in the majority of fermentations there was no excess of reduced products.

Since these results differ from those found by other investigators the accuracy of the analyses might be questioned but the carbon balance, as well as the redox index, shows that the analyses are, in general, satisfactory. The purity of the cultures is hardly to be questioned. Represented among the cultures studied are those received from four different investigators. It is evident that the present schemes of the propionic acid fermentation are not entirely satisfactory, inasmuch as it is impossible to apply experimental values to the schemes. The problem of the mechanism of the fermentation therefore requires further investigation.

Schemes of glucose dissimilation generally represent the hexose chain as being split into two 3-carbon molecules, an exception being Virtanen's 4- and 2-carbon cleavage. The 3-carbon scheme requires that every 2-carbon product be accompanied by a 1-carbon compound. It is evident that the CO_2 is not equivalent to the acetic acid in a number of fermentations. The large excess of CO_2 suggests the occurrence of a 2-carbon intermediary which is synthesised into a compound of higher number of carbon atoms. Succinic acid is a product which might thus originate. Each molecule of succinic acid formed would result in two molecules of CO_2 or some other 1-carbon compound. In some fermentations the quantities of CO_2 found are greater and in others less than that required by this hypothesis ($\text{CO}_2 \equiv \text{acetic} + 2x \text{ succinic acid}$). The occurrence of CO_2 in quantities greater than can be accounted for by the formation of succinic acid through a synthesis indicates there may be another source of CO_2 . The chemical structure of the non-reducing compound is not known, but there is apparently no correlation between it and the quantities of CO_2 produced. The suggestion is made that CO_2 originates in the formation of propionic acid. A decarboxylation of succinic acid would yield propionic acid and CO_2 .

It is realised that these proposals as presented are somewhat speculative. Supporting evidence will be given later, but to complete a possible scheme of the fermentation the results must be accounted for in which the yields of CO_2 are less than the calculated amounts when succinic acid is assumed to be formed by a synthesis. Two possibilities are suggested. The first is that succinic acid is formed by a 4- and 2-carbon cleavage of the glucose molecule thus yielding succinic acid without directly involving a 1-carbon compound. The second is that the succinic acid is formed by a synthesis and that part of the CO_2 (1-carbon compound) is utilised subsequent to its formation. Wood and Werkman [1936] presented evidence that CO_2 is utilised by the propionic acid bacteria during the fermentation of glycerol and pointed out that the evidence for Virtanen's 4- and 2-carbon cleavage is not conclusive. Although proof of the second suggestion cannot be considered conclusive until direct evidence is obtained that the propionic acid bacteria utilise CO_2 during the fermentation of glucose, our present information suggests such an occurrence. Possibly both the 4- and 2-carbon cleavage and CO_2 utilisation occur. Further investigation of this problem is under way.

It is not our purpose to claim in the present paper that the occurrence of the suggested reactions has been proved but to show that the data are in agreement with such a scheme. It is apparent that numerous reactions may give the same relative quantities of CO_2 , succinic acid *etc.*, as those involved in the formation of succinic acid from acetic acid. For example, the condensation of two molecules of pyruvic acid to diketoadipic acid with a subsequent splitting off of two molecules of CO_2 yielding succinaldehyde and oxidation of the succinaldehyde to succinic acid yields the same quantitative results.

Apart from the data there is evidence substantiating the formation of succinic acid by synthesis as shown by Wood and Werkman [1936]. The future problem is one of determining the intermediate compounds of the condensation. Evidence

of the activation of acetic acid has been obtained by the authors and these results are discussed in relation to this problem in a separate paper [Stone *et al.*, 1936].

The formation of propionic acid from succinic acid finds support in the authors' [1935] investigations showing that succinic acid occurs as an intermediary and may be decomposed. Hitchner [1934] obtained evidence that succinic acid is dissimilated to volatile acids in the presence of a simultaneous fermentation of carbohydrates. Shaw and Sherman [1923] presented data showing that their culture produced propionic and acetic acids in a solution of peptone and succinate; other investigators have not confirmed this, however. That the propionic acid bacteria can dissimilate succinic acid under certain conditions is definitely established but the mechanism and products of the dissimilation have not been determined. The dissimilation is represented as a decarboxylation. It is evident that the succinic acid could be destroyed by other reactions which yield CO_2 , thus giving the observed quantities of CO_2 . If the reaction proceeds by the Thunberg series and pyruvic acid is formed it is probable that part of the pyruvic acid will be oxidised to acetic acid and CO_2 . This oxidation would necessitate an increased reduction and thus an increase in propionic acid. The result would be an increase in CO_2 and propionic acid. For simplicity the change is shown as a decarboxylation in the following scheme (Fig. 1).

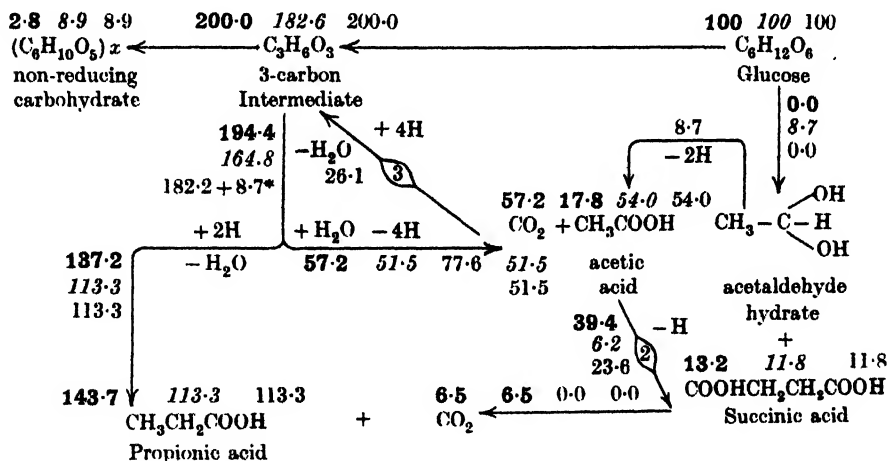


Fig. 1. Quantitative data and balances on substitution in the scheme of glucose dissimilation.
* 3-carbon compound from CO_2 .

Our results (Fig. 1) illustrate the agreement between the data and schemes involving the foregoing suggestions. The quantities of CO_2 , acetic acid and succinic acid have been used as a starting basis for substitutions. Fermentation 34W₁, Table II, may be used as an example in which the glucose may have been dissimilated entirely by a 3-carbon cleavage. In this case the formation of 17.8 m.mol. of acetic acid and 13.2 m.mol. of succinic acid would give rise to 44.2 m.mol. of CO_2 . 19.4 m.mol. of CO_2 are therefore unaccounted for. In the formation of propionic acid from succinic acid 3.0 m.mol. of CO_2 are formed for each m.mol. of propionic acid produced and 19.4/3 or 6.5 m.mol. of propionic acid would be produced from the succinic acid to give the observed value of CO_2 . 39.4 m.mol. of acetic acid are required for the 13.2 m.mol. of succinic acid and 6.5 m.mol. of propionic acid. 39.4 + 17.8 = 57.2 m.mol. of 3-carbon intermediate which would be dissimilated to acetic acid. The m.mol. of 3-carbon compound

corrected for the non-reducing material *minus* 57.2 gives the quantity of propionic acid produced from the 3-carbon intermediate. This quantity of propionic acid (137.2) *plus* that formed from succinic acid gives the calculated propionic acid (143.7). The experimental value is 139.1. Since the carbon recovery is not quite complete the calculated and observed values cannot be in perfect agreement. The calculated values of +H and -H are 274.4 and 268.2 respectively.

Fermentation 11W₉ may be cited as an example in which a 4- and 2-carbon cleavage may have occurred or a utilisation of CO₂. Consider first the 4- and 2-carbon cleavage (data in *italics*). If all of the acetic acid and succinic acid were produced following a 3-carbon cleavage of the glucose molecule there would be formed 77.6 m.mol. of CO₂. The observed value is 51.5. Each molecule of glucose fermented to succinic acid and acetic acid by the 4- and 2-carbon cleavage would decrease the quantity of CO₂ produced by 3 m.mol. 77.6 - 51.5 divided by 3 gives 8.7, the smallest quantity of glucose which could undergo a 4- and 2-carbon cleavage and give a calculated quantity of CO₂ equal to the observed. Using 8.7 as the glucose fermented by a 4- and 2-carbon cleavage, substitutions have been made as described above. The observed propionic acid is 114.8 and the calculated 113.3, +H = 226.6, -H = 229.6.

Calculations from the same fermentation are shown in which there is an assumed utilisation of CO₂. The method of calculation is evident. The observed propionic acid is 114.8 and the calculated 113.3, +H = 331.4, -H = 334.0.

It is evident that the scheme of fermentation meets the requirements of the quantitative data. Although a number of the fermentations do not give as good balances as the two chosen, they substantiate the scheme. The authors have purposely omitted showing intermediate reactions involved in the fermentation for the reason that present information does not warrant definite conclusions and because the intermediate reactions are not important to the calculations. No information is available as to the status of phosphoglyceric acid in this fermentation. The indicated synthesis of CO₂ to a 3-carbon intermediate is speculative.

At present it is impossible to explain accurately why results so markedly different from those of other investigators have been obtained in these fermentations. Culture 49W is a transplant of Van Niel's culture 4. Van Niel used an extract of 250 g. of yeast per litre of medium as a nitrogen source and his results indicate that a considerable quantity of fermentable material was included in the yeast extract. This is not the case with Bacto-yeast extract. After 30 days' fermentation at 30° with culture 34W in 0.4 % yeast extract and 1.4 % CaCO₃ there were produced 3.5 m.mol. of volatile acid, 4.7 m.mol. of CO₂, 0.3 m.mol. of succinic acid and no non-reducing sugar per litre of medium. Oxidation-reduction balances calculated from Van Niel's results are not satisfying and suggest that the nitrogen source materially affected the carbohydrate metabolism. It is difficult to speculate as to the mechanism of fermentation under such conditions. The use of various sources of nitrogen may have caused the different types of fermentation. The formation of succinic acid entirely from compounds of the yeast is excluded in our fermentation, for in exp. 36W₅ 5.2 g. of succinic acid were produced per litre in a medium containing 4.0 g. of yeast extract.

SUMMARY.

The propionic acid fermentation of glucose has been investigated quantitatively and the results show certain points of disagreement with present schemes of the fermentation. The following conversions are suggested to be involved and the data are shown to fit schemes including these reactions.

1. The formation of succinic acid by a synthesis, possibly by the condensation of two molecules of acetic acid.
2. The intermediate dissimilation of succinic acid causing an increased production of propionic acid and CO_2 .
3. A condensation of CO_2 and a subsequent utilisation of the synthesised product. The possibility is left that the 4- and 2-carbon cleavage occurs as suggested by Virtanen.

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LXXXIX. ACTIVATION OF THE LOWER FATTY ACIDS BY PROPIONIC ACID BACTERIA.¹

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SUCCINIC acid arising in the dissimilation of glucose by propionic acid bacteria has been regarded as resulting either from a 4- and 2-carbon splitting of the sugar molecule [Virtanen, 1923] or from the yeast extract in the medium [Van Niel, 1928]. Recently Wood *et al.* [1935] suggested its formation by condensation of two molecules of acetic acid followed by dehydrogenation. Assuming the latter reaction, resting cells of *Propionibacterium* should "activate" acetic acid, *i.e.* (cause a donation of hydrogen to a suitable acceptor, such as methylene blue [see Thunberg, 1920; Quastel and Whetham, 1924]. This study deals with the activation of acetic and related acids by *Propionibacterium*.

EXPERIMENTAL.

The resting cell technique of Quastel and Whetham was followed but, instead of timing the colour change, the progress of the reaction was followed by the redox potential [Yudkin, 1935]. The organisms were grown in a medium consisting of 0.5 % yeast extract (Difco), 0.5 % peptone, 1 % glucose and 0.2 % K_2HPO_4 . After 72 hours' incubation at 30° the cells were washed three times with sterile 0.85 % NaCl by centrifuging. One ml. of the paste-like centrifugate was diluted to 40 ml. with saline solution and stored at 6–8° not longer than 24 hours before use.

The experiments were carried out in pyrex tubes with side arms (Fig. 1), fitted with rubber stoppers, each carrying a KCl-agar electrode, a bright platinum electrode, a gas inlet tube and a gas outlet. A constant temperature water-bath at 37° was equipped to hold 16 of the redox tubes. After addition of the organisms (in the side cup), buffer and solutions, a stream of nitrogen, purified by passing over hot copper gauze and through alkaline pyrogallate, was bubbled rapidly through each tube for at least five minutes.

Reading of the initial potential was taken on the substrate-buffer mixture by use of the vacuum-tube amplifier described by Werkman, Johnson, and Coile [1933]; the organisms were then mixed with the contents of the tube and the mixture agitated. During the experiment a constant nitrogen pressure was

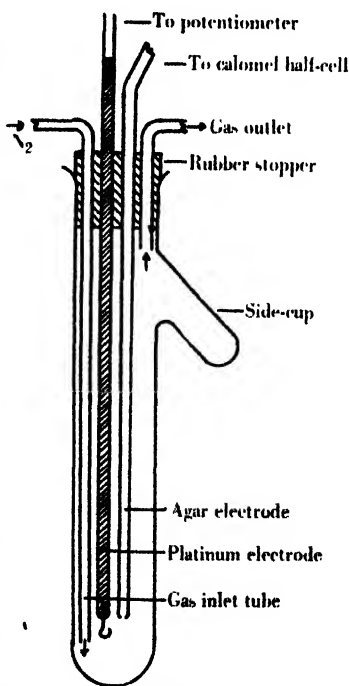


Fig. 1.

¹ This work was supported in part by Industrial Science Research Funds and the Rockefeller Fluid Research Fund of Iowa State College.

maintained in the tubes by means of a mercury trap. The course of the potentials was followed by constructing time-potential curves.

The tubes and electrodes were thoroughly cleaned with chromic acid. Electrodes were calibrated against each other by placing them in one large cell: only those reading within 2 mv. of the mean were used.

The substrate and buffer solutions were prepared with boiled redistilled water and the p_H taken with a glass electrode. During the experiment the p_H rarely changed more than 0.05 of a unit owing to the use of a relatively large quantity of buffer.

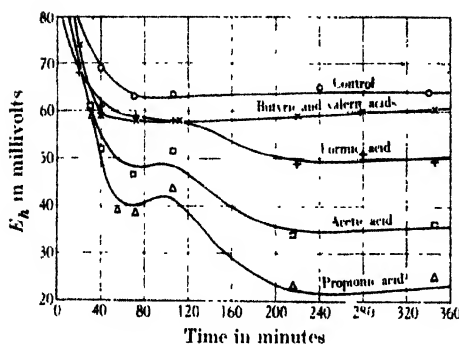


Fig. 2.

Fig. 2. ○ Control; × butyric and valeric acids; + formic acid; □ acetic acid; Δ propionic acid.

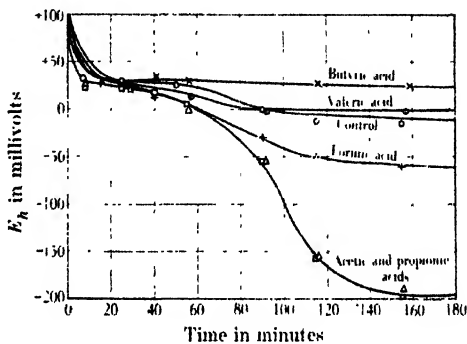


Fig. 3.

Fig. 3. × Butyric acid; ● valeric acid; ○ control; + formic acid; □ acetic acid; Δ propionic acid.

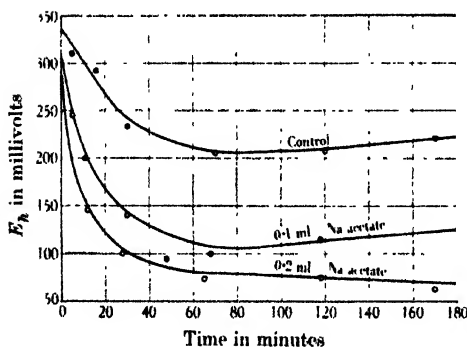


Fig. 4.

Fig. 4. 1st curve (top) control; 2nd curve (middle) 0.1 ml. Na acetate; 3rd curve (bottom) 0.2 ml. Na acetate.

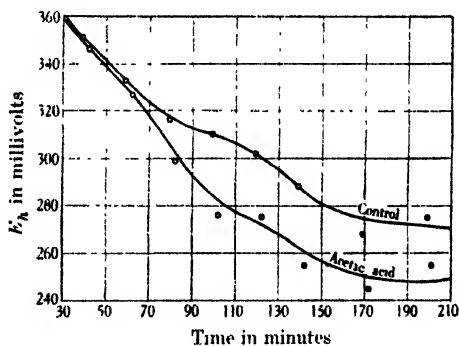


Fig. 5.

Fig. 5. Upper curve control; lower curve acetic acid.

Studies were carried out at 37° on formic, acetic, propionic, butyric and valeric acids using methylene blue and in some cases NaNO_3 , or *o*-chlorophenol-indophenol as hydrogen acceptor. Three species of *Propionibacterium* have been employed: *P. arabinosum* (34W), *P. pentosaceum* (49W), and *P. shermanii* (52W). These are described by Werkman and Brown [1933]. In a typical experiment 10 ml. of 1% phosphate buffer, 1 ml. of methylene blue (0.005 *M*), 0.2 ml. of 0.05 *M* substrate (Na salt) and 3.8 ml. of H_2O were placed in the redox

tube and 1 ml. of bacterial suspension in the side-cup. For the control tube 4.0 ml. of H_2O were added and the substrate omitted. Bacteria effecting dehydrogenation of the substrate bring about a reduction of methylene blue and a corresponding lowering of E_h . Representative time-potential curves are shown in Figs. 2-5, and additional results are condensed in Table I.

Table I. *Activation of lower fatty acids by Propionibacterium at 37°.*

Substrate	H-Accep.	Species	p_H 5.0-6.0			p_H 6.0-6.5			p_H 6.5-7.0			Total		
			+	-	?	+	-	?	+	-	?	+	-	?
Formic acid	M.B.	34W	1	0	0	0	0	1	3	1	1	8	5	2
	"	49W	0	1	0	1	0	0	2	2	0			
	"	52W	.	.	.	0	1	0	1	0	0			
Acetic acid	M.B.	34W	2	0	0	1	0	0	5	0	1	18	2	3
	"	49W	2	0	0	2	1	0	2	1	1			
	"	52W	.	.	.	3	0	1	1	0	0			
Acetic acid	$NaNO_3$	34W	1	0	0	.	.	.	2	.	.	7	1	0
	$NaNO_3$	49W	3	0	0	1	0	0	2	1	0			
	OCl	34W	.	.	.	2	0	0	2	0	0			
Propionic acid	M.B.	34W	1	0	0	1	0	0	5	0	0	14	3	1
	"	49W	1	0	1	0	1	0	3	0	0			
	"	52W	.	.	.	1	2	0	2	0	0			
Butyric acid	M.B.	34W	0	1	0	0	0	1	0	1	0	0	3	2
	"	49W	0	1	0	0	0	0	0	0	1			
Valeric acid	M.B.	49W	0	1	0	0	0	1	0	1	0	0	2	1

H-Accep. — Hydrogen acceptor. OCl — *o*-Chlorophenolindophenol. M.B. — Methylene blue.

NOTE. Figures in the columns marked + indicate the number of experiments showing activation under the conditions stated; — indicates no activation. Results showing slight or questionable activation are placed in the ? column.

DISCUSSION.

Formic acid shows activation under certain conditions; however, the reduction is not as rapid as in the cases of acetic and propionic acids. Clear-cut hydrogen donations are most frequently shown at p_H values near 7.0 (Fig. 3). The species of *Propionibacterium* show no marked individual differences in their behaviour towards formic acid. It may be pointed out that of the 5 negative results, 1 was obtained with organisms older than 72 hours, and 3 others had potentials higher than the control. We have found that aging causes inhibition of the dehydrogenases of *Propionibacterium*. It is possible that the high potentials may be explained by the sodium formate either inhibiting dehydrogenase activity or acting to some extent as a hydrogen acceptor. Apparently the propionic acid bacteria show a moderate attack on formic acid. This is of interest in relation to the observation of Wood and Werkman [1936] that CO_2 is utilised by the propionic acid bacteria in their dissimilation of glycerol. It is possible that the CO_2 is reduced to formic acid and that the formic acid is in turn assimilated.

Activation of acetic acid.

Propionibacterium generally activates acetic acid to donate hydrogen to methylene blue. Nitrates and *o*-chlorophenolindophenol also act as hydrogen acceptors (Figs. 4 and 5). The results with nitrate show a strong activation at p_H 5.0-6.0. At p_H values near 7.0 the reduction is not as marked. In experiments using $NaNO_3$ as a hydrogen acceptor, the potentials were not as stable as when a dye was added. However, though no added reversible system was present, a

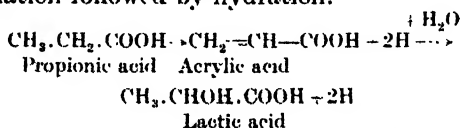
readable potential was set up, owing perhaps to some reversible system in the bacterial suspension.

The donation of hydrogen by acetic acid in the presence of *Propionibacterium* is of particular interest in view of the suggestion of Wood *et al.* [1935, see also Grey, 1924] that two molecules of acetic acid may condense to form one molecule of succinic acid along with two atoms of hydrogen. The hydrogen may, in the dissimilation of glucose, be accepted by pyruvic acid [Wood and Werkman, 1934] or some other hydrogen acceptor. The fact that the hydrogen donation to nitrates is particularly marked at an acid reaction is interesting when we consider that acetic acid tends to accumulate in the early period of the propionic fermentation (when the medium is less acid) and then decreases [Wood and Werkman, 1934].

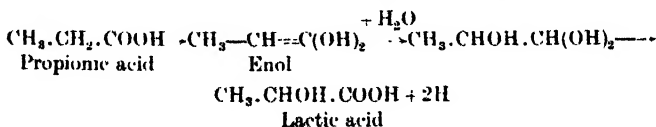
Activation of propionic acid.

The propionic acid bacteria cause a marked activation of propionic acid, particularly at p_H values near 7.0. Failures to activate occurred at lower p_H values. In general the rate of reduction of the dye by propionic acid approximates to that by acetic acid (Figs. 2 and 3). The significance of these observations cannot be determined definitely at the present time. Possibly activation is to be expected, since the organism produces the compound, and enzymic catalysis is by definition reversible. The conversion of lactic into propionic acid is readily brought about by *Propionibacterium* but the exact mechanism is not understood. If we assume that the reverse process takes place (*i.e.* propionic acid to lactic acid), we may postulate two mechanisms:

(1) Dehydrogenation followed by hydration.



(2) Enolisation followed by hydration and dehydrogenation.



In each of these reactions two hydrogen atoms are available and the reduction of methylene blue or other acceptor is accounted for. Thus in the fermentation of glucose, whether the dissimilation of propionic acid occurs would depend on the presence of a suitable hydrogen acceptor. Such a breakdown of the acid might go unnoticed if it proceeded *via* either of these reactions, for the resulting lactic acid may be isolated as an intermediary and is readily fermented. The possibility of propionic acid occurring as an intermediate product has apparently not been previously suggested.

Activation of butyric and valeric acids.

Neither butyric nor valeric acid showed any substantial donation of hydrogen to methylene blue. In the case of butyric acid, two of the results showed higher potentials than the controls (see Fig. 3). This may be the result of an inhibition of dehydrogenases by the acid.

SUMMARY.

A study with resting cells of *Propionibacterium* has been carried out on the lower fatty acids with three hydrogen acceptors by determining the E_h of the systems. The following observations have been made.

1. Acetic and propionic acids are shown to be activated by *Propionibacterium* to reduce methylene blue at p_H 5.0–7.0.

2. Formic acid is able to donate hydrogen to methylene blue to a small extent, whilst butyric and valeric acids show no such ability.

3. Acetic acid may donate hydrogen to nitrates and *o*-chlorophenol-indophenol.

These results are discussed in view of their significance to the propionic fermentation.

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XC. α -GLYCEROPHOSPHATE DEHYDROGENASE.

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MEYERHOF [1919] was the first to observe the oxidation of glycerophosphate by muscle and liver tissue. Ahlgren [1925], Quastel and Whetham [1925], Quastel and Wooldridge [1927], Alwall [1928: 1929], Collett *et al.* [1929], Davies and Quastel [1932] and McGavran and Rheinberger [1933] have considered some of the properties of the glycerophosphate dehydrogenase. There has not been however any systematic investigation of this enzyme, and there are no data available as to the method of preparation, the conditions for maximum activity, the nature of the oxidation product or the mechanism of the reaction with molecular oxygen.

I. *Preparation of the enzyme.*

The dissected skeletal muscles of a freshly killed rabbit were passed twice through a coarse meat mincer, and washed exhaustively with tap water. The washed mince was mixed with sand and 500 ml. distilled water and ground to a paste in a mechanical mortar. The sand and insoluble debris were filtered off through muslin. 50 ml. of *M*/10 acetate buffer of p_H 4.6 were added to the filtrate, and the precipitate was centrifuged. The supernatant fluid was discarded and the precipitate was resuspended in 100 ml. *M*/5 phosphate buffer of p_H 7.2. The enzyme suspension retains the bulk of its activity for a period of 10 days if kept at 0°. There is a definite fall in activity even at this low temperature. The precipitate can also be dried *in vacuo*. The enzyme in the dried form is quite stable.

II. *Reaction with methylene blue.*

The aerobic experiments were carried out in Barcroft manometers. The substrate was placed in dangling Keilin tubes and mixed with the enzyme after equilibration of the manometers.

Fig. 1 shows the effect of the methylene blue concentration on the rate of reaction of the substrate with molecular oxygen. There is no oxygen consumption by the enzyme suspension in absence of substrate. However, there is a definite, though small, uptake in presence of α -glycerophosphate without added carrier. The magnitude of this blank is variable and depends to a great extent on the thoroughness with which the minced tissue is both washed and ground. Saturation of the enzyme with methylene blue is reached at a concentration of about 7 mg. dye/3.3 ml. of fluid in the manometer cup. The low affinity of the glycerophosphate dehydrogenase for methylene blue resembles that of the hexosemonophosphate dehydrogenase of blood [Warburg and Christian, 1931].

Experiments on the anaerobic reduction of methylene blue were carried out in Thunberg tubes using the technique of Green and Dixon [1934]. The speed of reduction of methylene blue varied directly with the concentration of methylene blue (Table I).

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Table I. *The velocity of methylene blue reduction as a function of the concentration of methylene blue.*

2 ml. enzyme suspension, 0.2 ml. $M/10$ α -glycerophosphate, water to 5.2 ml. total volume.

$M/500$ methylene blue (ml.)	3.0	1.5	0.75	0.3	0.1	0.1 (without substrate)
Reduction time (min.)	32	18.5	14.5	9.5	6.0	∞
$100/t \times$ ml. methylene blue	9.36	8.10	5.18	3.15	1.66	—

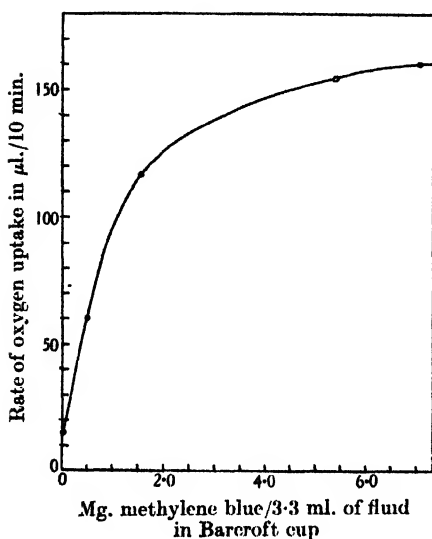


Fig. 1.

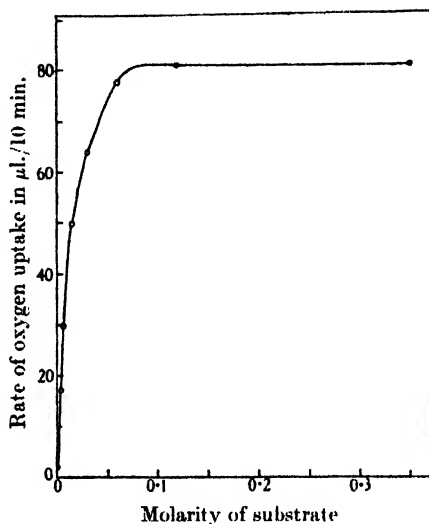


Fig. 2.

Fig. 1. *Effect of methylene blue concentration on the rate of oxygen uptake.* 1 ml. enzyme suspension, 0.2 ml. $2M$ ammonium α -glycerophosphate. Total volume of fluid 3.3 ml. p_H 7.2, 39°.

Fig. 2. *Effect of substrate concentration on the rate of oxygen uptake.* 1 ml. enzyme suspension, 0.3 ml. 0.5% methylene blue. p_H 7.2.

Table II. *Effect of concentration of substrate on rate of reduction of methylene blue.*

0.5 ml. enzyme suspension, 0.5 ml. 0.02% methylene blue, 1 ml. buffer p_H 7.2, water to 4 ml. total volume.

α -Glycerophosphate ml. $2M$ ml. $M/10$	2.0	1.0	0.5	0.25	0.10	—	—
Final molarity	—	—	—	—	—	0.2	0.05
Reduction time (min.)	10.0	9.5	9.5	9.0	8.0	9.5	12.4
$100/T$	10.0	10.5	10.5	11.1	12.5	10.5	8.0

III. Concentration of substrate.

The relation between the substrate concentration and the velocity of oxygen uptake is shown in Fig. 2. The Michaelis constant (K_M), i.e. the molar substrate concentration at which half the limiting velocity is reached, is 0.01. It is doubtful whether such a high concentration is ever present in animal tissues. This can mean either that the dehydrogenase in the cells does not function at maximum

velocity or that the Michaelis constant is not a fixed property of the dehydrogenase and depends upon the conditions of the experiment. Table II shows that the Michaelis constant is about 0.001 when the methylene blue concentration is reduced to 1/25 that in the manometric experiments.

IV. p_H .

In testing the dependence of reaction velocity on p_H the difficulty due to the natural buffering action of α -glycerophosphoric acid at neutral reaction was overcome, (a) by using reduced concentrations of substrate which were rather less than those required for the limiting velocity, (b) by using the enzyme so diluted that no appreciable change in the substrate concentration occurred during the course of the readings, and (c) by using concentrated buffer solutions. At the end of the various experiments no appreciable shift of p_H had occurred.

It was found that enzyme activity fell off rapidly below p_H 6 and above p_H 12 (Fig. 3). Within the limits of p_H 6–11 the velocity varied only slightly with hydrogen ion concentration. The variation is due more probably to differences in the buffer systems rather than to differences in p_H .

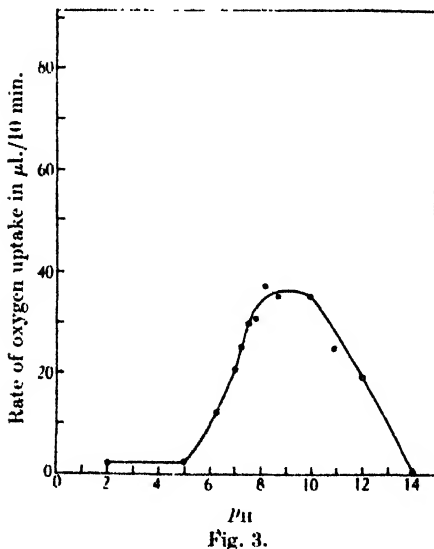


Fig. 3. Effect of p_H on the rate of oxygen uptake. 0.5 ml. enzyme suspension, 0.2 ml. 0.2 *M* substrate, 0.1 ml. 0.5% methylene blue, 2.5 ml. of *M* 5 buffer.

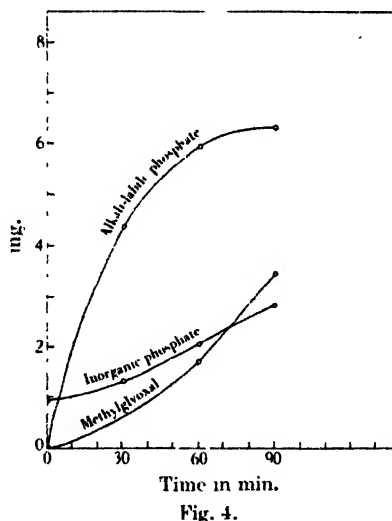


Fig. 4. The course of the oxidation of α -glycerophosphate.

V. Product of the oxidation of α -glycerophosphate.

200 ml. of enzyme suspension (representing the extract of two rabbits) were mixed with 50 ml. of 0.5% methylene blue and 100 ml. of *M*/10 α -glycerophosphate. Oxygen was passed through the mixture for 2 hours at 39° at such a rate as to maintain the methylene blue in a state of partial oxidation. Completion of the reaction was indicated by a sudden reoxidation of all the reduced methylene blue. After a further 10 min. treatment with O_2 , the enzyme suspension was deproteinised with 50 ml. of 50% trichloroacetic acid, filtered through filter-paper impregnated with kieselguhr to remove the methylene blue, and concentrated

in vacuo to 50 ml. Then 100 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl were added, and the mixture was kept at 70° for 10 min. The bulky red precipitate was filtered off and washed thoroughly with 2*N* HCl and finally with alcohol and ether. The yield of the crude product was 500 mg. The derivative was recrystallised several times from a mixture of amyl alcohol and nitrobenzene. The appearance of the crystals, solubility and colour in alcoholic potash corresponded with those of the 2:4-dinitrophenylosazone of methylglyoxal; m.p. 290° (uncorr.), authentic specimen m.p. 289°, mixed m.p. 289°. (Found (Weiler): C, 41.93; N, 25.61; H, 2.66; O, 29.8%. $C_{15}H_{12}O_8N_8$ requires C, 41.66; N, 25.92; H, 2.77; O, 29.6%.)

Johnson [1936] has recently isolated the 2:4-dinitrophenylosazone of methylglyoxal from a solution of α -glycerophosphate which had been incubated with minced brain tissue.

The question next arose whether methylglyoxal was a decomposition product of an unstable triosephosphate, presumably the first product of oxidation of α -glycerophosphate. Both the aldo- and keto-triosephosphates are extremely unstable in alkali, decomposing to yield lactic and phosphoric acids. This instability permits the quantitative determination of the triose ester simply by splitting off the phosphate group in *N* alkali, and estimating the inorganic phosphate [Meyerhof and Lohmann, 1934]. In the experiments that follow, inorganic phosphate has been estimated by the method of Fiske and Subbarow [1925], and methylglyoxal by the method of Ariyama [1928]. Triosephosphate does not interfere with the estimation of methylglyoxal.

The following typical experiment shows the course of the enzymic oxidation of α -glycerophosphate. 85 ml. of enzyme suspension (p_H 8) were mixed with 10 ml. *M*/10 α -glycerophosphate and 5 ml. of 0.5% methylene blue. The suspension was kept in a tall cylinder, immersed in a thermostat at 39°, and a rapid stream of O_2 was passed through. 10 ml. were pipetted out at intervals, deproteinised with trichloroacetic acid, decolorised by filtration through kieselguhr and analysed for inorganic phosphate, alkali-labile phosphate and methylglyoxal. The curves of Fig. 4 show that during the first 30 min. there was a large increase in alkali-labile phosphate although the production of methylglyoxal and inorganic phosphate was small. After 90 min., both inorganic phosphate and methylglyoxal showed large relative increases whereas triosephosphate was not greatly changed. An experiment at p_H 6.8 gave similar results (Table III) except that the rate of formation of inorganic phosphate and methylglyoxal was much slower.

Table III. *Course of the oxidation of α -glycerophosphate.*

Time min.	Inorganic P mg.	Alkali-labile P mg.	Methylglyoxal mg.
0	0.88	0.62	0
30	1.00	1.57	0
60	1.12	2.25	Trace
90	1.90	3.47	0.9

It is quite clear that the primary product of oxidation is a triosephosphate which decomposes in neutral solution to yield methylglyoxal and phosphoric acid. The appearance of methylglyoxal takes place only after a considerable formation of triosephosphate and is invariably accompanied by an increase in inorganic phosphate. The correspondence between methylglyoxal and inorganic phosphate formed is not exact owing to the partial conversion of methylglyoxal into other products.

Numerous attempts were made to isolate the triosephosphate as the barium salt following the procedure of Meyerhof and Lohmann [1934]. Decomposition of the triosephosphate during isolation was however so considerable that the final product contained only a small amount of the original alkali-labile phosphate.

It was found that the triosephosphate could be stabilised with KCN, which is usually a reagent for fixing an aldehyde group. 40 ml. of enzyme suspension, 35 ml. of *M*/10 α -glycerophosphate, 15 ml. of 2% NaHCO_3 , 15 ml. of 0.5% methylene blue and 5 ml. of *M* KCN were mixed together. The mixture was aerated and samples were drawn for analysis after various intervals. Table IV

Table IV. *Effect of KCN on the course of the oxidation of α -glycerophosphate.*

Time min.	Inorganic P mg.	Alkali-labile P mg.	Methylglyoxal mg.
0	0	0	0
70	Trace	24.0	0
120	"	27.8	0
180	"	27.8	0

shows that when the triosephosphate is fixed with cyanide no inorganic phosphate or methylglyoxal is formed. The barium salt of the triosephosphate-cyanohydrin was isolated in the following way. 100 ml. of enzyme (representing the extract of the muscles of 2 rabbits) were mixed with 100 ml. *M*/10 α -glycerophosphate, 15 ml. of *M* NaCN (neutral), 25 ml. of 0.5% methylene blue and 50 ml. of 2.5% NaHCO_3 . After 2 hours' aeration of the mixture 20 ml. of 50% trichloroacetic acid were added and the precipitate filtered through kieselguhr. The clear, colourless filtrate was cooled to 0°, neutralised with NaOH until just acid to bromthymol blue and mixed with 40 ml. of 25% barium acetate. Cold acetone was added in equal volume and the precipitate was centrifuged. The supernatant fluid was mixed with an equal volume of cold alcohol and the second precipitate centrifuged. The two precipitates were washed thoroughly with alcohol and ether and dried *in vacuo*. The first precipitate weighed 2.1 g. and the second 0.9 g. The latter usually contained a higher percentage of triosephosphate. The two precipitates were found to be mixtures of the barium salts of triosephosphate-cyanohydrin, α -glycerophosphate and a small amount of inorganic phosphate. Triosephosphate accounted for 50–60%, inorganic phosphate for 0–10%, and difficultly hydrolysable phosphate for the balance of the total phosphate.

Labidity of the phosphate group in alkali does not sufficiently identify a triosephosphate. The presumed triosephosphate should also yield an equivalent quantity of methylglyoxal and phosphoric acid in *N* HCl at 100° [Meyerhof and Lohmann, 1934]. Table V shows that the crude barium salt, dissolved in dil. HCl

Table V. *Production of methylglyoxal and phosphoric acid from crude Ba triosephosphate after acid hydrolysis.*

Barium salt (dried *in vacuo* at 80°), 92 mg.

	Alkali-labile P (mg./92 mg. barium salt)	Methylglyoxal (mg./92 mg. barium salt)
Initial	0	0
15 min. in <i>N</i> alkali at 18°	2.50	—
1 hour in <i>N</i> HCl at 100°	2.45	4.20

Observed methylglyoxal = $\frac{4.2}{5.8} = 72\%$ theory. Observed P = $\frac{2.45}{2.50} = 98\%$ theory.

and decomposed with H_2SO_4 has this property. Attempts to purify the barium salt were unsuccessful. Fractional precipitation with acetone and alcohol failed to yield a product with more than 3% alkali-labile P. Apparently the solubilities of barium triosephosphate-cyanohydrin and barium α -glycerophosphate are very similar. An insoluble derivative formed in the cold with 2:4-dinitrophenylhydrazine was the osazone of methylglyoxal. The ready decomposition of triosephosphate in presence of this reagent thus explains the large yield of methylglyoxal bishydrazone in the early isolation experiments. Fischer and Baer [1932] have prepared the actual 2:4-dinitrophenylhydrazone of glyceraldehyde-phosphate by rapid manipulation at low temperatures, but state that large amounts of the methylglyoxal derivative were also formed. *p*-Bromophenylhydrazine and *p*-nitrophenylhydrazine similarly yielded the derivative of methylglyoxal. Unsymmetrical methylphenylhydrazine formed a derivative under conditions in which methylglyoxal did not react. The product however was oily and defied crystallisation.

The triosephosphate may be either glyceraldehydephosphate or dihydroxyacetonephosphate. The aldotriose has been synthesised by Fischer and Baer [1932] and its properties studied in more detail by Meyerhof and Lohmann [1934]. Kiessling [1934] has improved the synthesis of dihydroxyacetonephosphate and studied its chemical properties. Apparently both trioses have an alkali-labile phosphoric group and decompose in *N* HCl at 100° to yield methylglyoxal and phosphoric acid. The velocity constants of hydrolysis are practically identical. The only method distinguishing between them is based on the fact that the aldotriose reacts with I_2 or Br_2 whereas the ketotriose is unaffected by these reagents. By use of this method Meyerhof and Lohmann [1934] and Meyerhof and Kiessling [1935] established that dihydroxyacetonephosphate is the principal scission product of hexosediphosphate.

The titration of triosephosphate with I_2 involves certain difficulties. The reaction must be carried out in alkaline solution, as a result of which some methylglyoxal is formed and oxidised by the iodine. Since the ketotriose may similarly liberate methylglyoxal the iodine titration offers no final evidence of the presence of an aldotriose; it should therefore be controlled by determination of inorganic and alkali-labile phosphate. If the iodine is utilised to oxidise the aldotriose to the corresponding acid, for each molecule of I_2 absorbed 1 molecule of alkali-labile phosphate should disappear without reappearing as inorganic phosphate. Phosphoglyceric acid is extremely resistant to hydrolysis and its phosphorus would not be estimated. If the iodine is utilised to oxidise methylglyoxal, an equimolecular amount of alkali-labile phosphate should be replaced by inorganic phosphate. In other words, iodine titration proves the presence of an aldotriose only when the alkali-labile phosphate is simultaneously converted into a more difficultly hydrolysable form.

Although Meyerhof and Kiessling [1935] recommend that the reaction with I_2 should be carried out in Na_2CO_3 in order to minimise spontaneous decomposition, we were compelled to use 0.1*N* NaOH in order to dissociate the cyanohydrin.¹ Even so, oxidation was not quite complete in 1 hour. 10 ml. of a solution of triosephosphate (equivalent to 20 mg. Ba salt) were neutralised and mixed with 1 ml. *N*/10 iodine and 5 ml. of *N*/10 NaOH. A control with no triosephosphate was also prepared. After 30 min. 1 ml. of 6*N* HCl was added to the control and experimental flasks and the iodine titrated with *N*/100 thiosulphate. The results are given in Table VI.

¹ The presence of cyanide in the Ba salt was shown by the Prussian blue test. The triosephosphate-cyanohydrin solution must be heated with NaOH before the test is applied.

Table VI. *Oxidation of triosephosphate by iodine.*

Before iodine		Thiosulphate back-titration		After iodine		Increase of inorganic P mg.	Decrease of alkali-labile P mg.	% P which is no longer estimated
Inorganic P mg.	Alkali-labile P mg.	Control ml.	Exp. ml.	Inorganic P mg.	Alkali-labile P mg.			
0.045	0.500	10.05	4.35	0.189	0.087	0.144	0.413	65
0.045	0.500	10.00	4.40	0.193	0.073	0.148	0.427	65

Many such experiments confirm that no more than 35% of the alkali-labile phosphate which has disappeared can be accounted for in the inorganic phosphate fraction. The evidence definitely points to the formation of phosphoglyceric acid. Admittedly the iodine titration was always substantially higher than corresponded with the loss in alkali-labile phosphate; but it is well known that in strong alkali the stoichiometric proportions do not hold and that the equivalents of iodine vary from 2 upwards according to the conditions.

Apparently, then, the oxidation product of α -glycerophosphate is principally and perhaps entirely glyceraldehydephosphate. There is however no evidence definitely excluding small amounts of ketotriose. The complete chemical description of the triosephosphate must be deferred until it can be isolated in a pure state.

The ease with which methylglyoxal can be formed from the triosephosphate supports the contention of the Meyerhof and Embden schools that methylglyoxal occurs only as a decomposition product in carbohydrate metabolism, and is in no sense an intermediary metabolite.

VI. *The number of oxygen equivalents.*

For this work pure α -glycerophosphoric acid is essential. We have therefore further purified our calcium α -glycerophosphate (Merck, D.A.B. 6, stated by Meyerhof and Kiessling [1933] to be practically pure α -salt) by Rae, Kay and King's [1934] modification of Karrer and Salomon's [1926] method for separating the α - and β -isomerides. The final barium salt obtained contained 40.70% Ba, corresponding with $C_3H_5O_6P\text{Ba}, 2H_2O$.

Fig. 5 shows that exactly one-half the theoretical amount of oxygen for the complete oxidation of α -glycerophosphate to glyceraldehydephosphate is taken up. Presumably only one of the two optical isomerides in the synthetic (α) product is oxidised by the enzyme system. Since the chemical resolution of the optical isomers of α -glycerophosphate is difficult to confirm [Rae *et al.*, 1934] owing to the small magnitudes of the rotations, it is not possible to examine each isomeride separately. However, a natural product of muscle glycolysis [Embden *et al.*, 1933; Meyerhof and Kiessling, 1933] is available which consists of only one optical isomeride since it yields twice as much lactic acid as the synthetic substance when incubated with the muscle glycolytic enzyme and pyruvate. Through the courtesy of Prof. O. Meyerhof I have been able to test the action of the dehydrogenase on this natural α -glycerophosphate. Fig. 6 shows that the oxidation proceeds to completion and that the isomeride attacked by the dehydrogenase is identical with the one formed in glycolysis.

The optical specificity of the enzyme explains why complete oxidation of synthetic α -glycerophosphate was not obtained in the isolation experiments even with a large excess of enzyme. α -Glycerophosphate from muscle has been designated as (–) α -glycerophosphate [Meyerhof and Kiessling, 1933]. Smythe and Gerischer [1933] have shown that yeast ferments only the dextrorotatory

component of synthetic glyceraldehydephosphate. Since it is only this component of the synthetic triosephosphate which can be dismutated by muscle, it follows that $(-)$ α -glycerophosphate gives rise to $(+)$ glyceraldehydephosphate on oxidation.

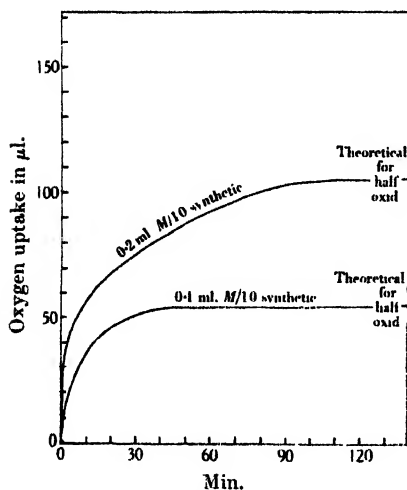


Fig. 5.

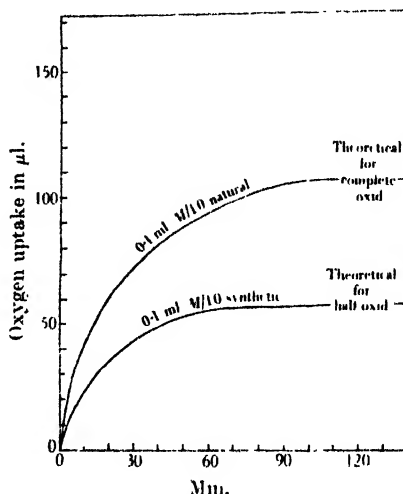


Fig. 6.

Fig. 5. The number of oxygen equivalents of synthetic α -glycerophosphate. 2.0 ml. enzyme suspension, 0.3 ml. 0.5% methylene blue, p_H 7.2. 0.3 ml. 10% KOH (in centre pot). The small control uptake has been subtracted from the experimental uptake.

Fig. 6. The number of oxygen equivalents of natural α -glycerophosphate. Details as in legend to Fig. 5.

VII. Specificity of donor.

Glycerol, β -glycerophosphate, synthetic 2-phosphoglycerate and natural 3-phosphoglycerate are not oxidised by the muscle dehydrogenase. The test experiments must be carried out manometrically since traces of α -glycerophosphate present in these substances are sufficient to reduce a small quantity of methylene blue in presence of the enzyme though insufficient for any appreciable oxygen uptake. The specimen of β -glycerophosphate was obtained from Boot's Research Department through the courtesy of Dr Pyman. It was 94% pure β -salt. Synthetic 2-phosphoglyceric acid was prepared by the method of Kiessling [1935]. Natural α -glycerophosphate was kindly given by Prof. C. Neuberg.

VIII. Specificity of acceptor.

In addition to methylene blue, all reversible indicators with an E'_0 at p_H 7 of greater than -0.2 v. can be reduced by the α -glycerophosphate dehydrogenase. The speed of reduction depends to a great extent upon the number of acidic groups, and is generally lower the more acidic the compound. Benzylviologen can be reduced only in alkaline solution ($p_H > 8.5$). This suggests that the potential level of the α -glycerophosphate system lies near that of the lactic system, *i.e.* about -0.25 v.

Nitrate can be reduced to nitrite but only extremely slowly. This contrasts with xanthine oxidase which even when quite weak will reduce a fair quantity of nitrate in presence of hypoxanthine [Bernheim and Dixon, 1928]. A very active α -glycerophosphate enzyme is required to produce in several hours an

amount of nitrite which is just detectable by the sensitive Griess-Illosvay reagent. The two controls (1) containing all the components without substrate, and (2) containing α -glycerophosphate and no enzyme, were negative.

The dehydrogenase system does not reduce pyruvate or fumarate (as indicated by failure to form alkali-labile phosphate). The disappearance of pyruvate was also looked for analytically but there was no indication of any reaction. Thus the enzyme which catalyses the reaction between α -glycerophosphate and pyruvate in muscle glycolysis is distinct from the dehydrogenase which activates α -glycerophosphate for oxidation.¹ There is other evidence supporting this view. The active glycolysing muscle juice does not contain any α -glycerophosphate dehydrogenase and conversely the dehydrogenase preparation cannot catalyse any of the glycolytic processes. Furthermore, 0.001 *M* iodoacetate which according to Meyerhof and Kiessling [1933] inhibits the reaction between α -glycerophosphate and pyruvate has no effect on the oxidation of glycerophosphate (Section X). Johnson [1936] claims that the Embden-Meyerhof scheme does not apply to brain since he was unable to obtain any reaction between α -glycerophosphate and pyruvate in minced brain tissue. But it is clear from his results that he was studying the activity of α -glycerophosphate dehydrogenase which as shown above is not directly concerned with glycolysis. The possibility still remains open that in the intact brain the Embden-Meyerhof scheme may apply in part.

IX. *Competitive inhibition.*

It is generally accepted that there are two distinct processes involved in the oxidation of a substrate by the appropriate dehydrogenase. The first involves adsorption on the enzyme of some group of the substrate molecule: the second involves activation of the adsorbed molecule and consequent oxidation. The enzyme usually exhibits a high degree of specificity in the second process. But many substances structurally similar to the particular substrate oxidised are capable of being specifically adsorbed on the enzyme surface although they are not activated. The simplest method of demonstrating specific adsorption is that of competitive inhibition. Adsorption on the enzyme surface of a substance which is not activated interferes with the oxidation of the proper substrate. The two substances compete for the active groups of the dehydrogenase. Fig. 7 shows the inhibition of the oxidation of α -glycerophosphate by 3-phosphoglycerate. 2-Phosphoglycerate similarly inhibits the oxidation though not to as marked an extent. β -Glycerophosphate has no action at all.

X. *Respiration inhibitors.*

Table VII shows the effects of KCN and NaN_3 on the rate of oxygen uptake. Both reagents increase the velocity of oxidation when present in concentrations sufficient to poison completely the respiration of animal cells and yeast.

Table VII. *Effect of KCN and NaN_3 .*

2 ml. enzyme suspension, 0.2 ml. 2*M* α -glycerophosphate, 0.3 ml. 0.5% methylene blue, water to 3.3 ml. total volume.

<i>M</i> /10 KCN (ml.)	—	0.3	—
<i>M</i> /10 NaN_3 (ml.)	—	—	0.5
$\mu\text{l. O}_2$ /10 min.	155	210	191
% acceleration	—	36	20

¹ These experiments were done in collaboration with D. D. Woods.

Table VIII demonstrates the inhibitory effects of ethylurethane and octyl alcohol. Those results agree with the findings of Keilin [1925; 1929] that cyanide, CO and NaN_3 inhibit primarily the indophenol oxidase, whereas narcotics like urethane and octyl alcohol inhibit the dehydrogenases.

Table IX shows that both NaF and iodoacetate have no appreciable effect on the oxygen uptake in concentrations which poison glycolytic processes.

Table VIII. *Effect of narcotics.*

1.5 ml. enzyme suspension, 0.2 ml. 2*M* α -glycerophosphate, 0.3 ml. 0.5% methylene blue, water to 3.3 ml. total volume.

5% ethylurethane (ml.)	—	1.0	—
Octyl alcohol (ml.)	—	—	0.3
$\mu\text{l. O}_2/10 \text{ min.}$	95	70	36
% inhibition	—	26	63

Table IX. *Effect of NaF and $\text{CH}_2\text{I.COOH}$.*

1.0 ml. enzyme suspension, 0.2 ml. 2*M* α -glycerophosphate, 0.3 ml. 0.5% methylene blue.

	$\mu\text{l. O}_2/10 \text{ min.}$	% inhibition
Control	59	
<i>M</i> /600 iodoacetate	59	0
<i>M</i> /300 iodoacetate	47	20
<i>M</i> /60 NaF	49	17
<i>M</i> /30 NaF	41	30

XI. *Production of H_2O_2 .*

Dehydrogenases are for convenience divided into two groups—those that react directly with molecular oxygen (*e.g.* xanthine, uric acid and amino-acid oxidases) and those that require an intermediary carrier. Keilin and Hartree [1936] have demonstrated clearly that the aerobic oxidases as a class produce H_2O_2 , and that the H_2O_2 may be used in the presence of alcohol and catalase to oxidase alcohol to aldehyde. Detection of H_2O_2 by this oxidation of alcohol serves as a useful means of deciding whether the oxygen uptake of a dehydrogenase preparation is due to a direct reaction with molecular oxygen or whether the blank reaction with oxygen is catalysed by traces of cytochrome. If the yellow pigment is present however, this argument does not hold since the yellow pigment produces H_2O_2 on autoxidation.

Ogston and Green [1935] considered the α -glycerophosphate enzyme to belong to the class of aerobic oxidases since the blank reaction with oxygen of their cruder preparation was not removed by exhaustive washing and was hardly influenced by the addition of respiratory carriers. However, when the enzyme is extracted by the method described in Section I, the blank reaction though present is relatively weak. Table X shows that the addition of catalase

Table X. *Effect of catalase and alcohol.*

Enzyme suspension (ml.)	2.0	—	2.0	2.0	2.0
Liver catalase (ml.)	—	0.7	0.7	—	0.7
<i>M</i> alcohol (ml.)	—	0.25	0.25	—	0.25
α -Glycerophosphate 2 <i>M</i> (ml.)	0.2	0.2	0.2	—	—
α -Glycerophosphate 0.1 <i>M</i> (ml.)	—	—	—	0.2	0.2
Water (ml.)	1.1	2.15	0.15	1.1	0.15
$\mu\text{l. O}_2/15 \text{ min.}$	28.4	1.4	28.2	—	—
$\mu\text{l. O}_2/110 \text{ min.}$	—	—	—	54.8	51.6

and ethyl alcohol does not either increase the speed of oxidation or affect the total uptake, both of which should be increased were H_2O_2 produced. Another additional line of evidence against the production of H_2O_2 is the fact that the theoretical uptake for the oxidation of α -glycerophosphate was reached. If H_2O_2 were formed, the final value would be twice the theoretical.

On the basis of this and subsequent evidence it is probable that the α -glycerophosphate enzyme is of the anaerobic type and that in absence of a proper carrier it does not react directly with molecular oxygen.

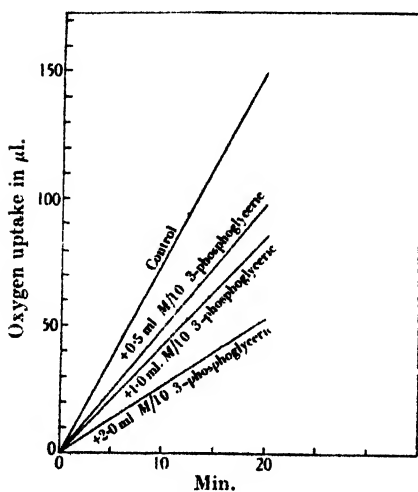


Fig. 7.

Fig. 7. Inhibition by 3-phosphoglyceric acid. 0.5 ml. enzyme suspension, 0.3 ml. 0.5% methylene blue, 0.1 ml. 2M α -glycerophosphate. p_{H} 7.2.

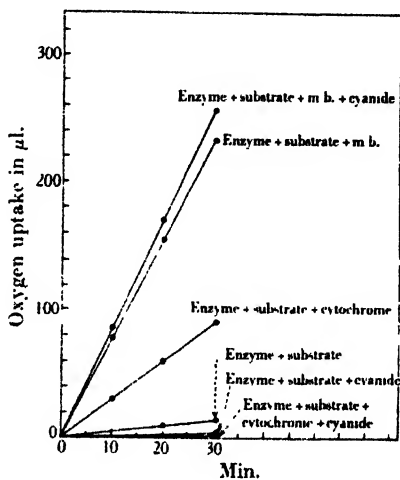


Fig. 8.

Fig. 8. Effect of heart cytochrome c and of methylene blue on the reaction of the dehydrogenase with molecular oxygen. 1 ml. enzyme suspension, 0.2 ml. 2M α -glycerophosphate. Final concentrations: cytochrome = 0.15×10^{-4} M, methylene blue = 0.13×10^{-2} M; cyanide = M/600.

XII. Intermediary carriers.

Tables XI to XIII show that glutathione, Warburg and Christian's flavo-protein, lactoflavin, adrenaline and ascorbic acid, cannot function as respiratory carriers for the enzyme system. The autoxidation of ascorbic acid is in fact inhibited by the enzyme preparation, as was first observed with tissues by Green [1933]. This protective action can be eliminated by addition of methylene blue (Table XIV); this is probably due to metallic impurity in the methylene blue preparation.

Table XI. Effect of flavoprotein, oxidised glutathione and lactoflavin.

	$\mu\text{l. O}_2/15 \text{ min.}$	$\mu\text{l. O}_2/30 \text{ min.}$
Control (enzyme + substrate)	30	61
Control + 16 mg. flavoprotein	28	59
Control + 4 mg. GSSG	27	56
Control + 8 γ lactoflavin	30	60
Control + 5 mg. methylene blue	290	550

Table XII. Effect of adrenaline.

	$\mu\text{l. O}_2/30 \text{ min.}$
Control (enzyme + substrate)	24
Control + 1 mg. adrenaline	33 (without enzyme, 9)

Table XIII. *Effect of ascorbic acid.*

	$\mu\text{l. O}_2/10 \text{ min.}$
Control (enzyme + substrate)	4
Control + 5 mg. ascorbic acid	15 (without enzyme, 61)

Table XIV. *Reversal of protective action of enzyme on autoxidation of ascorbic acid.*

	$\mu\text{l. O}_2/10 \text{ min.}$
5 mg. ascorbic acid	58
Enzyme suspension	0
Enzyme + 5 mg. ascorbic acid	7
Enzyme + 5 mg. ascorbic acid + 1 mg. methylene blue	264

Fig. 8 contrasts the actions of heart cytochrome c^1 and methylene blue on the oxidation of α -glycerophosphate, and the effect of cyanide on the respective catalyses. $0.15 \times 10^{-4} M$ heart cytochrome c catalyses the oxidation of α -glycerophosphate with a speed slightly less than half that in presence of $13 \times 10^{-4} M$ methylene blue ($= 87 \times$ cytochrome concentration). KCN completely inactivates the cytochrome system whereas it increases the methylene blue effect. Cytochrome c of yeast behaves in similar fashion (Fig. 9): cyanide completely inhibits the catalysis.

The catalytic activity of cytochrome has been shown by Keilin [1925; 1929] to depend on two factors: the dehydrogenase system which reduces cytochrome, and the indophenol oxidase which oxidises the reduced form. There should therefore be an active indophenol oxidase in the α -glycerophosphate preparation in order to account for the cytochrome effect. Table XV shows that such is actually the case. The effect of cyanide is therefore entirely on the indophenol oxidase.

Table XV. *Indophenol oxidase in α -glycerophosphate preparation.*

	$\mu\text{l. O}_2/20 \text{ min.}$
Enzyme suspension	0
Enzyme + 5 mg. <i>p</i> -phenylenediamine	189 (without enzyme, 5)

Ogston and Green [1935] were unable to detect any appreciable effect of yeast cytochrome c on the oxidation of α -glycerophosphate by muscle brei. Thus it appears that the ratio of cytochrome to dehydrogenase is far greater in the brei than in the enzyme preparation. Table XVI shows that a high concentration of cytochrome c produces less than a threefold increase in the velocity of oxidation by muscle brei as compared with a thirteenfold increase when the same concentration is added to the enzyme preparation. The effect of cytochrome c on the oxidation by the brei falls off rapidly with dilution. Thus little effect of cytochrome can be demonstrated unless a high concentration ($1 \times 10^{-4} M$ and greater) is used.

¹ I am indebted to R. Hill and Prof. D. Keilin for allowing me to use their unpublished elegant method for the preparation of heart cytochrome c . The concentration of cytochrome c was determined by comparing colorimetrically unknown cytochrome solutions with standard solutions of mesohaemin pyridine haemochromogen, the absorption spectrum of which is practically identical with that of cytochrome c of both yeast and heart. The colorimetric determinations were checked from time to time by spectrophotometric determinations. For the latter the absorption coefficients determined by Dixon *et al.* [1931] were used.

Table XVI. *Comparison of cytochrome effect on muscle brei and on enzyme preparation.*

	Chopped muscle		Enzyme preparation	
	$\mu\text{l. O}_2/20 \text{ min.}$	$\%$ increase	$\mu\text{l. O}_2, 20 \text{ min.}$	$\%$ increase
α -Glycerophosphate	31	—	9	—
+ 0.5 ml. yeast cytochrome $1 \times 10^{-4} M$	86	277	120	1330

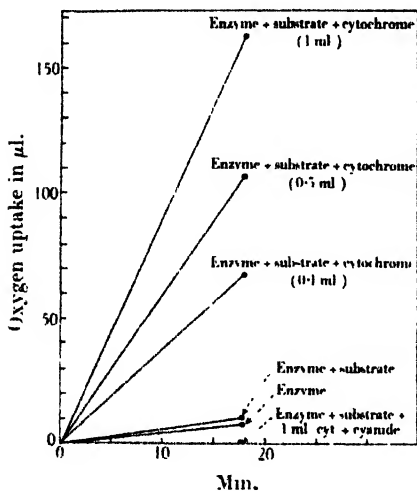


Fig. 9.

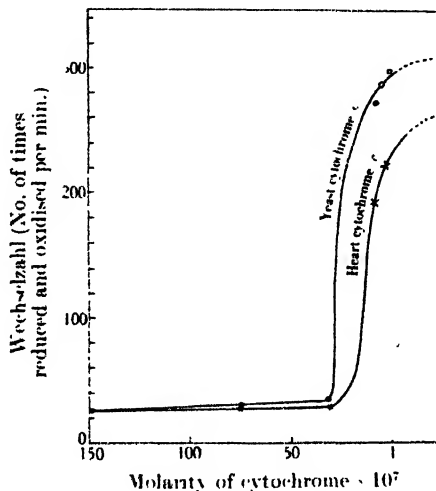


Fig. 10.

Fig. 9. Effect of varying concentration of yeast cytochrome *c* on the oxygen uptake. 1 ml. enzyme suspension, 0.2 ml. 2 *M* α -glycerophosphate. Cyanide = *M* 600. Added cytochrome, $1 \times 10^{-4} M$.

Fig. 10. The dependence of the "Wechselzahl" on the molar concentration of cytochrome *c*. 1 ml. enzyme, 0.2 ml. 2 *M* α -glycerophosphate.

$$\text{Wechselzahl} = \frac{\text{oxygen uptake, min.}}{\text{oxygen equivalent of added cytochrome}} \quad \text{---} \quad \text{Period of observation, 10 min.}$$

Warburg and Christian [1933] introduced the interesting concept of "Wechselzahl" or turnover number which they defined as the number of times per min. each molecule of a carrier is both reduced and oxidised. It is the ratio of the total oxygen uptake/min. to the oxygen equivalence of the carrier. Cytochrome of living yeast according to Haas [1934] and Warburg [1934] reaches a value of 4000. Yeast cytochrome *c* has a value of 35 when catalysing the oxidation of succinic or lactic acid [Ogston and Green, 1935].

Obviously the limiting value of the "Wechselzahl", determined with the carrier working at full capacity, will be the most important value. This is best determined by gradual dilution of the carrier until the effect almost disappears. Since the rate of reduction of a given amount of cytochrome *c* will depend upon the amount of enzyme present,¹ the greatest rate per molecule will be reached at infinite dilution of the cytochrome. In practice, the increase in rate due to very minute quantities of carrier falls within experimental error, and it is only by extrapolation that the limiting value can be reached.

¹ This reasoning does not hold if the affinity of the enzyme for cytochrome falls off rapidly with dilution of cytochrome.

Fig. 10 shows the effect of dilution on the turnover numbers of heart and yeast cytochrome *c* when catalysing the oxidation of α -glycerophosphate. At the higher concentrations the oxygen uptake is nearly proportional to cytochrome concentration and the number is almost constant (about 25). Below 3.5×10^{-6} *M* extensive dilution of cytochrome produces only a small decrease in the extent of catalysis; hence the number steadily increases and reaches about 310 as the limiting value for yeast cytochrome *c* and about 270 for heart cytochrome *c*. The close correspondence of the curves for the two cytochromes offers good evidence of their chemical identity.

The precise meaning of the turnover number requires some clarification. The number is a property not only of the carrier but of the reducing and oxidising systems. If hydrosulphite is added to cytochrome the reduction is practically instantaneous. Suppose that an oxidant like ferricyanide could be introduced, which would react only with reduced cytochrome. Then both the oxidation and reduction of cytochrome would be practically instantaneous and the turnover number would approach infinity. Suppose, however, that the indophenol oxidase replaces ferricyanide as oxidant. The turnover number will then depend upon the speed of oxidation of reduced cytochrome by the indophenol oxidase. There is a limit to the speed of oxidation which is determined by molecular sizes and shapes, and the probability of collision of the three components of the system, *viz.* reduced cytochrome, indophenol oxidase and molecular oxygen. Therefore the turnover number is not a fixed property of either the carrier or the reducing and oxidising systems but is a property of the system as a whole. In the above experiments, the most active enzyme is the indophenol oxidase. Hence the turnover number refers to the catalytic activity of the reducing system, dehydrogenase-cytochrome, and not to the activity of the oxidising system.

It follows from this discussion that calculations of the proportion of the total respiration which proceeds through flavoprotein or cytochrome cannot be based merely on turnover numbers *in vitro*. This number being a function of the concentration of enzyme, spatial configuration *etc.* will obviously be different *in vivo* from that *in vitro*, and even *in vivo* will vary with the different dehydrogenase systems.

XIII. *Distribution of the enzyme in tissues.*

The various tissues of a freshly killed rabbit were minced, washed exhaustively with tap water and then ground with sand in a mortar. The brei was filtered through muslin, and the filtrate mixed with acetate buffer, p_H 4.6. The precipitates were centrifuged and resuspended in distilled water. Two experiments were carried out with the enzyme preparation of each tissue: one a control with all the components except the substrate, the other with all components present. In most cases the control was negative. In the calculation, the control respiration if present is subtracted from the experimental. The absolute value of the Q_{O_2} depends upon the concentration of methylene blue and substrate. These were therefore kept constant in all the experiments.

Table XVII. *Distribution of α -glycerophosphate dehydrogenase.*

$Q_{\alpha\text{-glycerophosphates}}$ *i.e.* O_2 uptake/mg. dry weight of enzyme/hour in presence of 1.5 mg. methylene blue. 38°.

Brain	3.82	Liver	2.66	Lung	1.43
Muscle	2.72	Kidney	2.42	Intestine	0.88
Heart	0.79				

Table XVII contains the various Q_{α} -glycerophosphate values. Brain has the highest and heart the lowest concentration of the tissues studied. It is curious that skeletal muscle should be much richer in this enzyme than heart muscle.

XIV. Purification of the enzyme.

In the preparation of the muscle enzyme, the acetic acid precipitate is re-suspended in phosphate buffer. On standing, the precipitate settles, leaving a clear supernatant fluid. If the mixture is centrifuged rapidly for 10 min., the supernatant fluid shows no enzyme activity (Table XVIII). Presumably the enzyme is attached to particles which are not soluble. Table XVIII also shows that the insolubility of the α -glycerophosphate enzyme holds also for brain and kidney.

Table XVIII. Loss of activity of enzyme suspensions after centrifuging.

	$\mu\text{l. O}_2/10 \text{ min.}$		
	Muscle	Brain	Kidney
Enzyme + α -glycerophosphate + methylene blue	61	255	70
Centrifuged 1 min.	36	—	—
Centrifuged 2 min.	35	—	—
Centrifuged 10 min.	0	—	—
Centrifuged 30 min.	—	38	4

XV. Coferments.

The method of preparation of the enzyme involving exhaustive washing of the tissue prior to extraction rules out the possibility of a soluble coenzyme. Neither cozymase nor the hexosemonophosphate coferment has any effect on the speed of oxidation of α -glycerophosphate. The possibility still remains that there is a coenzyme which is rigidly fixed to the enzyme and which is not removed by merely washing the particles to which the enzyme is attached.

XVI. Partial pressure of oxygen.

In all the manometric experiments, air was used as the source of oxygen. The enzyme system does not function any more rapidly at higher partial pressures of oxygen.

XVII. Yeast enzyme.

Ogston and Green [1935] prepared an active α -glycerophosphate dehydrogenase from yeast after grinding in a ball mill. This yeast preparation has the following significant properties. (1) The yeast enzyme requires a high concentration of methylene blue for maximum activity. (2) Synthetic α -glycerophosphate is only half-oxidised whereas (—) α -glycerophosphate is oxidised to completion. (3) The enzyme is soluble and cannot be removed from solution by centrifuging. (4) No success attended the attempt to link the enzyme with the cytochrome-indophenol oxidase system.

SUMMARY.

The preparation of an active α -glycerophosphate dehydrogenase from muscle has been described. The influence of p_{H} , methylene blue concentration, substrate concentration and partial pressure of oxygen on the rate of enzymic oxidation has been studied.

The dehydrogenase oxidises only (—) α -glycerophosphate, the natural muscle isomeride. Evidence has been presented that glyceraldehydophosphate is the principal oxidation product. The possibility that dihydroxyacetonephosphate is also formed is not excluded. Methylglyoxal arises as a decomposition product of the triosephosphate.

The enzyme is not inhibited by KCN or NaN_3 but is inhibited by octyl alcohol and ethylurethane. NaF and iodoacetate in concentrations which poison glycolytic processes have no action on the dehydrogenase.

The dehydrogenase does not catalyse the reaction between α -glycerophosphate and pyruvate. Evidence is presented that the dehydrogenase is not the enzyme responsible for this reaction in the glycolytic cycle.

Flavoprotein, flavin, ascorbic acid and adrenaline cannot catalyse the reaction of the dehydrogenase system with molecular oxygen. Cytochrome *c* of heart and yeast are the only common natural carriers which are active with this system. The cytochrome effect was analysed and it was shown that, under suitable conditions, cytochrome is reduced and oxidised about 300 times per min. The dehydrogenase is considered to be anaerobic in the sense that it does not react with oxygen in absence of a suitable carrier.

The enzyme is widely distributed in animal tissues, and in the rabbit is found in the highest concentration in the brain. When extracted from tissues, it is found associated with particles and cannot be brought into solution.

It is a pleasure to thank Miss J. Brosteaux for her kind assistance with the experiments on cytochrome and on the distribution of the enzyme. I am also indebted to Dr E. Friedmann for many suggestions.

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XCI. THE USE OF VARIOUS RAT ASSAY METHODS IN COMPARING CRYSTALLINE VITAMIN B₁ PREPARATIONS.

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IN continuation of the study of the parallelism between the ultraviolet absorption spectra of samples of vitamin B₁ preparations and their potencies [Heyroth and Loofbourow, 1932], three samples of crystalline material have been subjected to detailed assay. The diet of the rats and the symptoms produced have already been described in detail [Heyroth, 1932]. Considerable variation has been encountered in the time required before the symptoms become evident. No attempts have been made to determine the causes of this variation by altering the diet. When the symptoms became marked, the vitamin preparation in aqueous solution was injected subcutaneously and the subsequent developments noted. The weight changes, the number of days required to produce a cure and the duration of the cure were recorded. If the rat appeared normal 72 hours after the injection and was found paralysed 96 hours after injection, the cure was said to have persisted 3 days.

Three preparations were tested. (A) was preparation 64:19 of Prof. R. A. Peters, Oxford. (B) was oryzanin from yeast and (C) was oryzanin from rice polishings. (B) and (C) were prepared by S. Ohdake, Tokyo, and furnished through the courtesy of Prof. U. Suzuki and Dr Ohdake. To all these workers I wish to extend my thanks for making these preparations available.

(A) consisted of 2 ml. of a solution containing 1.19 mg. of crystals in 96% alcohol at p_H 3 (approx.). Peters has reported [Peters and Philpot, 1933; Kinnersley *et al.*, 1933] its pigeon day dose as 2.2 γ . (B) and (C) were furnished in crystalline form and were dissolved to make stock solutions subsequently diluted to strengths suitable for injections and for spectrographic work elsewhere reported [Heyroth and Loofbourow, 1936].

RESULTS.

Minimum curative dose.

Previous data of others with an early Windaus preparation [Windaus *et al.*, 1932; Seidell and Smith, 1930; Heyroth and Loofbourow, 1932, p. 37] suggested that the rat would require several times the pigeon day dose. For this reason the first doses of Peters's preparation employed were 12, 11 and 9 γ . The results showed that these were needlessly large; however, they furnished some information as to the effects of large doses. The following observations were made as a basis for a minimum curative dose of (A).

Three trials of 1.1 γ failed to cure. 1.95 γ failed in one trial. 2.25 γ failed in two trials, gave incomplete or very slow cures in two trials and produced delayed but complete cures in two trials. 3 γ cured in two trials. In a moribund rat, which may be neglected, death occurred shortly after injection. 4 γ produced

two cures. In a third rat traces of leg weakness persisted, but this rat was abnormal in that the paralysis was flaccid rather than spastic. All doses of 5 γ or more produced cures.

It therefore appears that the minimum rat curative dose of this preparation lies between 2.25 and 3 γ . This neglects the improvement noted in some rats which received doses of 2.25 or 1.5 γ . Since Peters reported the pigeon day dose of this preparation as 2.2 γ , the factor for converting the pigeon day dose into the rat minimum dose is 1.2.

In the case of the two Japanese preparations no attempt was made to determine the minimum curative dose, since we wished to make every injection yield some quantitative value. As the smallest dose of the yeast preparation used (3.97 γ) cured in several trials, the minimum curative dose must be less than this and so close to that of (A). Minimum curative doses have since been published by Ohdake and Yamagishi [1935], who give 3 γ for each. This value possibly represents a very slightly lower potency than that of (A).

Mean day dose.

This has been determined for each of the preparations and affords direct comparison of the needs of the pigeon and the rat for identical crystalline specimens (sample A).

In determining the time of cure it is necessary to adopt a definite criterion as to the return of the deficiency symptoms. We have employed usually the return of severe symptoms in which the paralysed rat not only is unable to walk but is so incoordinated that on attempting to right itself after having been placed on its back it rolls over completely, usually suffering convulsions during the attempt. Such a state is commonly preceded by two or three days during which there are very slight symptoms, most frequently a spastic state of one hind leg. It is obvious that if the appearance of such slight symptoms be adopted as the criterion of the end of a cure, the day dose will be higher. In the case of the first preparation assayed, only the severe symptoms were employed with sufficient regularity to make the results of value. With the Japanese preparations, more attention was paid to this point, so that the results are equally trustworthy whether the appearance of slight or of severe symptoms be taken as marking the end of the cure. There has also been employed as a third criterion of the end of the effect of the injection the loss of weight gained shortly after injection.

Whichever criterion was employed, certain of the values were regarded as questionable. These represented either resistant rats which remained cured for extremely long periods, rats which died from undetermined causes before the return of symptoms, or rats in which the symptoms were of an unusual type, e.g. flaccid rather than spastic paralysis. The mean values of all injections, and the means with the questionable cases eliminated (marked corrected) are given separately in Table I.

Table I. *Summary of the rat day doses in γ of three preparations.*

Preparation	Mild symptoms		Severe symptoms				Weight maintenance			
	All results	Corrected data	All results	σ as %	Corrected data	σ as %	All results	σ as %	Corrected data	σ as %
(A) Peters, 64 : 19	—	—	0.452 \pm 0.14	64	0.411 \pm 0.086	36	0.539 \pm 0.144	46	0.551 \pm 0.144	37
(B) Ohdake, yeast	0.717	0.600	0.531 \pm 0.119	53	0.509 \pm 0.085	35	0.774 \pm 0.24	66	0.713 \pm 0.232	63
(C) Ohdake, rice	0.675	0.508	0.604 \pm 0.284	91	0.450 \pm 0.114	44	0.843 \pm 0.506	108	0.588 \pm 0.192	51

Table II. *Comparative potencies of three preparations, that of Peters taken as 100.*

Preparation	Severe symptoms		Weight maintenance	
	All results	Corrected data	All results	Corrected data
(A) Peters, 64 : 19	100.0	100.0	100.0	100.0
(B) Ohdake, yeast	85.1	80.7	69.6	77.3
(C) Ohdake, rice	74.8	91.3	63.9	93.7

From the results of 14–22 injections for each preparation, the standard mean deviations (σ) have been computed for the severe symptoms and weight maintenance doses.¹ In Table I these have been presented as % of the mean value in order to afford a more ready comparison of the deviations found when employing the various criteria for the end of the cure. To each mean there has been appended 2ϵ , where ϵ is the standard error of the mean, $\epsilon = \frac{\sigma}{\sqrt{n}}$.

The weight criterion has the advantage of requiring less experience of the symptoms, but it demands a somewhat larger number of injections, since in some cases the symptoms recur before the weight has returned to its value at the time of injection. No attempt has been made to employ this criterion in rats declining in weight but not yet showing paralysis. It is open to the serious objection that the presence of other members of the vitamin B complex might lead to error, although in the present series there is no indication that such has been the case.

DISCUSSION.

There has been much discussion as to the validity of the results of the day dose method. Peters [Kinnersley *et al.*, 1935], who has employed it extensively, regards it as not very satisfactory for the crystalline vitamin in the pigeon, whilst Birch and Harris [1934] think that, properly employed, it is satisfactory. Two points require consideration, the question as to whether the size of the dose influences the value of the day dose and the great deviations of the results.

Previous workers [Kinnersley *et al.*, 1935; Van Veen, 1932; Coward *et al.*, 1933; Birch and Harris, 1934] have found that the duration of cure does not increase in direct proportion to the size of the dose, so that the day dose method can be safely employed only over a very limited range of duration of cure. The potency of a given preparation may appear less if large doses are employed than if smaller ones are used.

For the present series, these considerations are of relatively little importance, since the preparations were so nearly alike in potency that it would have been futile to attempt to select different dose levels for each to make the lengths of cure the same. Most of the doses injected were 4–5 γ , and the average time for the recurrence of severe symptoms was about 12 days. Only in the case of Peters's preparation were a few doses of 9 γ or more employed. If the higher doses should give higher day doses they would make this preparation appear less active in comparison with the other two. Therefore the inclusion of these few higher doses in the case of this preparation tends to reinforce the evidence that this preparation is slightly more potent than the others.

To test the validity of the view that the duration of cure influences the results, the data were divided into two groups in which the cures were (a) over and (b) under 10 days. For each preparation, using all values, there were computed the standard deviations from the means, as well as σ in % of the means.

¹ $\sigma = \sqrt{\frac{\sum d^2}{n-1}}$. The number of observations varied from 12 to 19.

For (A) they were 68 % for the low and 49 % for the high group. For (B) they were 36 and 40 %, and for (C) 84 and 47 %, respectively. The view of Birch and Harris [1934] that the deviations are somewhat greater with lower than with higher duration of cure is borne out.

To determine whether the order of activities of the three preparations is influenced by the duration of cure the means, with questionable values omitted, were compared in each of these two groups. The results are for the group with longer cures: (A), 0.384 γ ; (B), 0.398 γ ; (C), 0.391 γ . For those with shorter cures they are: (A), 0.484 γ ; (B), 0.710 γ ; (C), 0.634 γ . The order is the same in both groups, and it is shown in Table I that it is also the same when the results are not so grouped. The differences in the case of the group with longer cures, where the uniformity is also greater, are not statistically significant, which tends to lessen somewhat the significance of the differences found in the ungrouped results. No attempt was made to group the results by sizes of doses injected, since only a very limited range of doses was employed.

The magnitude of the deviations is of interest. Table I indicates that for all values this varies from 64 to 91 % of the means. With the omission of abnormal cases, this is lessened to about 35–44 %. In the pigeon, Peters usually found the deviation to be 44 %, while the results of Ohdako and Yamagishi [1935] appear somewhat more uniform as they calculate it to be 20–30 %. In the case of the weight criterion the results in (C) were worse. Such deviations greatly limit the value of curative methods in the intact animal for the comparison of crystalline preparations of a high degree of purity.

Histological studies [Hofmeister, 1922; Prickett, 1934] of the brains of rats showing the deficiency symptoms have shown disseminated foci of haemorrhage or intense congestion in the pons and medulla involving important cranial nerve nuclei. Peters and Thompson [1934] have found an impaired ability of the deficient pigeon's brain to oxidise pyruvic acid. The cure of the metabolic disturbance by the vitamin may be obscured by the presence of the complicating anatomical lesions. The large deviations encountered in the durations of the cures may have their origin in individual anatomical variations. The measurement of the oxygen uptake of minced brain, as in the catatorulin test of Peters, would be expected to approximate to the metabolic effects of the vitamin more closely.

Some speculation as to the reason for the dependence of appetite on the vitamin may perhaps be permitted from the histological work of Prickett [1934]. Lesions occurring in the regions of the nuclei of the fifth and ninth nerves might well destroy or lessen the sense of taste. Disturbances of other cranial nerves, *e.g.* exophthalmos, and more rarely nystagmus, have been encountered in the series.

To determine whether the differences in activity of the preparations indicated by the means in Table I are statistically significant, for each pair of means the sums of their variances, $\frac{\sigma^2}{n}$, have been divided into the differences of the means. If these quotients are large numbers, the differences may be judged significant. These values, from symptom criteria, are 27 for the (A)-(B) pair, 8 for the (A)-(C), and 12 for the (B)-(C). By the weight test they are respectively 9, 0.5 and 7. While these results are not large numbers, it is believed that they are sufficient, except in the case where weight was the criterion in the pair (A)-(C), to indicate that (A) has a significantly greater potency than (B); the deviations in the case of (C) were greater and it is not possible to be certain as to its comparative rating beyond the fact that it is not more potent than (A). In

Table I comparative values are given for the three preparations on the basis of 100% for (A). Although, as has just been indicated, its significance is limited, it is included for comparison with spectrographic data in the following paper.

The variations in the weights of the rats employed at the times of injection were not great enough to permit a conclusion as to the influence of the weight of the individual upon the dose required. In the few instances in which heavier rats were used, the day doses obtained were among the lowest, and any weight influence may have been masked by the great deviations encountered in the entire series.

When, however, the rat and the pigeon are compared, the interesting result is obtained that the day dose is proportional to the mean weights of the two species. In an earlier paper Peters *et al.* [1928] gave the weights of 17 pigeons at the onset of symptoms as between 205 and 310 g., the mean being 254 g. This agrees with the mean of 255 g. reported by Windaus *et al.* [1932] for 16 pigeons. The mean weight of our rats in the assay of Peters's preparation was 44 g. Multiplication of the dose for this preparation found by Peters for the pigeon by the ratio of the weights of the animals ($2.2\gamma \times 44/254$) gives 0.39γ . The rat day dose was found to be 0.41γ (Table I). The mean weight of Ohdake's pigeons [Ohdake and Yamagishi, 1935] at the time of injection was 207 g., and of our rats used in assaying his preparations, 44 g. Ohdake's pigeon day dose for either the yeast or the rice preparation was 2.4γ . (The identity of these preparations with the specimens sent to us is uncertain.) The calculated rat day dose is 0.51γ . There were found 0.509γ (yeast) and 0.450γ (rice). This fails to confirm the observations of Kinnorsley *et al.* [1930] that the day doses are the same for the pigeon and rat, irrespective of the difference in weights of the two species. Nor does it confirm the view of Cowgill [1934] that relative to body weight the rat requires more vitamin B₁ than the pigeon. (Cowgill, however, appears to have used as his criterion of activity growth for the rat and maintenance of a weight level for the pigeon.) Should man also have the same requirement, the curative day dose would be 0.6 mg. On the basis of Cowgill's expressions, a man of 154 lb. requires 122 times as much of a given vitamin preparation as does a pigeon of 254 g. Since the pigeon day dose for the prevention of the recurrence of severe symptoms is 2.2γ , the man would require about 0.27 mg. per day, or about half the amount calculated upon the assumption that weight for weight the demands of the pigeon and of man are equal.

From Ohdake's data [Ohdake and Yamagishi, 1935], the pigeon appears to differ in one respect from the rat. He reports the minimum pigeon curative dose as 3γ and the pigeon day dose as 2.4γ . In previous work in this laboratory [Heyroth and Loofbrourow, 1932], the minimum curative dose for the rat was found to be 6.6 times the curative day dose. This is confirmed in the case of Peters's preparation, since 6.6 times the day dose of 0.41γ would give 2.7γ as the minimum curative dose. Direct determination gave its value as between 2.25γ and 3γ . If this same ratio applied in the case of the pigeon, the minimum curative dose for that species would be about 16γ . The minimum curative doses seem about the same for the two species whilst the day doses stand in the ratio of their mean weights.

Since the rat day dose is 0.185 times that for the pigeon and the minimum curative dose for the rat is 6.6 times as great as the day dose, it follows that the minimum dose for the rat is 6.6×0.185 , or 1.2 times the pigeon day dose. This confirms the statement of Williams and Eddy [1931-32] that the minimum curative dose for the rat is certainly not less than the pigeon day dose, a statement based on the observation that prophylactic feeding tests on pigeons showed that

0.25 g. of rice polishings daily protects the pigeon indefinitely; 1 g. of the polishings yielded on thorough extraction with alcohol 3–5 curative doses.

It may also be noted that Ohdake found 2.3 γ as the curative day dose of his preparations when given orally and about the same value or 2.4 γ when given by injection. Kinnersley *et al.* [1935] believed a given sample to be 30 % more active by injection than when given orally. Van Veen [1932] found that preparations which were ineffective for the rat when given orally cured when injected. All doses in the present series have been injected.

SUMMARY.

1. Three samples of crystalline vitamin B₁ have been assayed by the rat method. Their potencies are, in the order of decreasing activity: Peters (64 : 19). Ohdake (rice), Ohdake (yeast). The differences between them appear to be only slightly beyond the limits of accuracy of the assay. The variations indicated should not be taken as an implication that the vitamin has not been isolated, but only as suggesting a contamination with slight but different amounts of impurities. Peters has recorded other samples of somewhat greater activity.

2. The minimum curative dose for the rat is 1.2 times the pigeon day dose, and the rat minimum curative dose is 6.6 times the day dose required for preventing the recurrence of severe symptoms.

3. In determining the curative rat day dose a definite criterion must be adopted as marking the return of symptoms. Different values are found when this is taken as the first spasticity of the hind leg, or as convulsions and inco-ordination. The order of activity of different preparations is the same in either case.

4. As a third criterion, giving still different numerical values, there may be employed the number of days which elapse before the rise of weight following the injection has been lost. This assumes the preparations to be uncontaminated by other vitamins of the B group.

5. Histological reasons are advanced to account for the rather large standard deviations encountered in these assays.

6. In the cases of rats and pigeons, the day doses required to prevent the recurrence of severe symptoms are proportional to the mean weights of the animals of the two species at the times of injection.

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XCII. FURTHER DEDUCTIONS AS TO THE CHEMICAL NATURE OF VITAMIN B₁ FROM ULTRA-VIOLET ABSORPTION SPECTRA.

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IN 1932 we presented a comparison of the ultra-violet absorption spectra and biological assay values of a series of the most active vitamin B₁ concentrates then available [Heyroth and Loofbourow, 1932]. That work indicated the vitamin to have an absorption maximum at about 2600 Å. and led us to suggest that it might contain a pyrimidine derivative or one of ergothioneine. Several crystalline preparations have since been made available to us through the kindness of Dr M. I. Smith, of the National Institute of Health, Prof. R. A. Peters, Oxford, Dr A. G. Van Veen, Batavia, Java, and Profs. Suzuki and Ohdake, of Tokyo, some of whom [*e.g.* Peters and Philpot, 1933; Ohdake, 1932] have published data on the ultra-violet absorption of this vitamin. As in our earlier work, we first attempted to correlate the ultra-violet extinction values at various wave-lengths with the biological potencies of the preparations.

The study of these newer preparations indicated for them an absorption curve with two peaks at 2360 and 2650 Å. suggestive of that of cytosine [Heyroth and Loofbourow, 1934, 1], rather than a single peak at 2600 Å. The publication by Peters and Philpot [1933] of a curve with a single peak at 2470 Å. caused us to delay publication of our curves except in a preliminary note [Heyroth and Loofbourow, 1934, 2] until a cause of the discrepancy could be found. With three of the newer preparations we found very satisfactory agreement between absorption in aqueous solutions and biological potency, but in view of the unexpected variations in the absorption of the vitamin, we must regard this as in part the result of coincidence.

Recently Windaus *et al.* [1934] demonstrated the presence of two ring compounds, one a pyrimidine, in the vitamin. The other has been identified by Clarke and Gurin [1935] as a thiazole derivative. Recent spectrographic studies by Williams *et al.* [1935] on the pyrimidine and by Ruehle [1935] on the thiazole component afford a basis for a reconciliation of our curves for the vitamin (which have been confirmed by Wintersteiner *et al.* [1935]) with those of Peters and Philpot [1933] and Holiday [1935]. We therefore present, in leaving this field, our absorption curves and analyse them in terms of the separate contributions made, under varying conditions, by each of the two rings of the vitamin.

Absorption curves of crystalline vitamin B₁ of various workers.

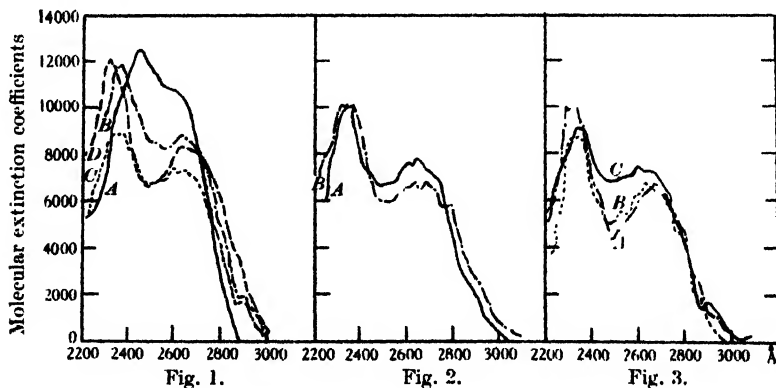
The first crystalline samples made available to us (April, 1933) were three picrolonates of the vitamin isolated by Dr A. Seideli [Seidell and Smith, 1933] and furnished through the courtesy of Dr M. I. Smith, who also provided us with a solution which he had assayed of the vitamin hydrochloride, prepared

in the laboratory of Prof. Peters. It was thought to have been his preparation 53:A14.

From the absorptions of these picrolonate solutions it was necessary to deduct the absorptions of the amounts of picrolonic acid assumed from the biological potencies of the solutions to be present. The resulting curves for the absorption of the vitamin differed from the curve previously published by Windaus *et al.* [1932] in exhibiting two well-defined absorption bands with maxima at about 2350–2400 Å. and 2650–2700 Å. separated by a minimum at about 2550 Å. A certain measure of correlation between extinction and biological activity was found over the range 2600–2800 Å. The result indicated that the wave-length (2600 Å.) at which we had previously found the closest correlation does not correspond exactly with that of one of the maxima of the more highly purified vitamin.

The hydrochloride preparation of Prof. Peters in general confirmed this new type of absorption curve (Fig. 1, D). In addition to the maximum at 2650 Å. there is a stronger one situated at 2330 Å. This curve differed from the curves obtained from the picrolonate solutions only in having a more marked minimum.

Shortly after we had obtained this curve, Peters and Philpot [1933] presented absorption curves of a number of samples which differed both from that reported by Windaus *et al.* [1932] and from that which we had just found in having one somewhat asymmetrical band with its maximum between 2450 and 2500 Å. To aid in elucidating the discrepancy, Prof. Peters kindly sent us a solution of his sample 64:19 in alcohol about $N/200$ in hydrochloric acid. Repeated determinations of the absorption of this sample diluted with about 25 to 50 volumes of either water or alcohol showed that the solvent was not responsible for the discrepancy (Fig. 1). Van Veen's preparation gave the same curve with water as with alcohol as solvent (Fig. 2). This curve agreed in general with the more recent ones of Wintersteiner *et al.* [1935], in both solvents.



Vitamin B₁ hydrochlorides.

Fig. 1. Preparations of Peters: A— 64:19 in water; B— 64:19 in alcohol; C— 64:19 in water; D— 53:A14 in water.

Fig. 2. Preparations of Van Veen: A— in water; B— in alcohol.

Fig. 3. A— Average of two yeast oryzanin curves; B— Average of two rice-oryzanin curves; C— Preparation 64:19 of Peters in water.

Fig. 1 indicates also a lack of reproducibility of the absorption curves in water; our curve in alcohol also differs from that of Holiday [1935], who has attributed the variations to changes in p_H between 5.5 and 7.0. It is unlikely,

however, that the vitamin undergoes tautomeric change with alteration of p_H since the curves of Peters and Philpot [1933] under extremely varied conditions of acidity and alkalinity failed to exhibit the two-banded type of absorption.

Holiday [1935] also thought that when dissolved in acid alcohol various samples of the vitamin, including recent ones of Windaus and Van Veen, gave absorption of the Peters and Philpot type with considerable uniformity. Before the appearance of his paper we had also found a similar uniformity in the two-banded absorption of aqueous and alcoholic solutions of preparations by Ohdake (Fig. 3, A and B) and Van Veen (Fig. 2), which agreed with the curves of Seidell's samples and with those of Peters (Fig. 3, C) under certain conditions, as well as with those later reported by Wintersteiner *et al.* [1935]. In 1934 we published a note [Heyroth and Loofbourow, 1934, 2] comparing the absorptions of Ohdake's preparations (Fig. 3, A and B) from rice polishings and from yeast with that of one curve of Peters 64 : 19 (Fig. 3, C). The rat day doses of these three preparations were found to be [Heyroth and Loofbourow, 1936] of the order of 100, 85, 91 for the (64 : 19) yeast and rice preparations, respectively. The molecular extinction values at 2600 Å. were found to be 7400, 5700, 6200, in very close agreement with this ratio of potencies. However, had we chosen one of the other curves for Peters's preparation (Fig. 1), the agreement would have been less apparent. It is obvious that the variability of the absorption of the vitamin greatly lessens the value of any correlation of potency and absorption of this material, unless the comparison be restricted to curves of the same form.

The limitations of biological assay render the slight variations in potency of crystalline preparations, as at present prepared, somewhat uncertain, and this, together with the variations in their absorption, makes any agreement between potency and absorption appear fortuitous. The general agreement that the crystals obtained by most workers represent the very nearly pure vitamin, moreover, makes unnecessary the continuation of such studies.

Interpretations of the two types of vitamin B₁ ultra-violet absorption curves.

In a communication to Prof. Peters cited by Holiday [1935], Windaus has stated that his more recent preparations yield curves of the Peters-Philpot type. It remains now to correlate the recent work on the constitution of the vitamin with its ability to exhibit either one-banded absorption as found by Peters and Philpot or the two-banded curve first observed by us.

After cleavage of the vitamin by the action of sodium sulphite, Williams *et al.* [1935] isolated two products, one of which they believed to be the sulphonic acid of an ethyl- (or dimethyl-) aminopyrimidine. After deamination it yielded the sulphonic acid of an ethyl- (or dimethyl-) oxypyrimidine.

Very recently, Clarke and Gurin [1935] have identified the sulphur-containing ring as 4-methyl-5- β -hydroxyethylthiazole and Williams [1935] views the vitamin as a quaternary compound of the thiazole with a chloride of the aminopyrimidine. Ruehle's curves [1935] for the absorptions of the free thiazole and its quaternary methiodide and those for the pyrimidine [Williams *et al.* 1935] now afford a basis for making tentative pyrimidine-thiazole summation curves, which indicate a probable explanation of the variations in absorption exhibited by the vitamin.

Two processes require consideration, a deamination of the vitamin, analogous to that demonstrated by Williams *et al.* [1935] for the pyrimidine component, and the breaking of the quaternary linking resulting in a dissociation of the vitamin into its components, a change shown by Williams to occur in the

presence of sodium sulphite which forms a relatively insoluble pyrimidine sulphonc acid.

To prepare summation curves covering various stages of these two possibilities, there are required the absorptions of the tertiary and quaternary forms of the thiazole and those of the free and bound oxy- and amino-pyrimidines, six curves in all.

The absorption of the tertiary thiazole has been given by Ruehle [1935]; his curve for the thiazole methiodide may be employed as an approximation to the contribution to the vitamin absorption made by the thiazole when in quaternary combination with the pyrimidine.

The vitamin curves of Peters and Philpot [1933] and of Holiday [1935] in acid alcohol agree in the location of their maxima with that of the absorption of the sulphonc acid of the aminopyrimidine, and may be considered as summations of the absorptions of the quaternary thiazole with that of the aminopyrimidine when bound to thiazole. Deduction of the curve of the thiazole methiodide from

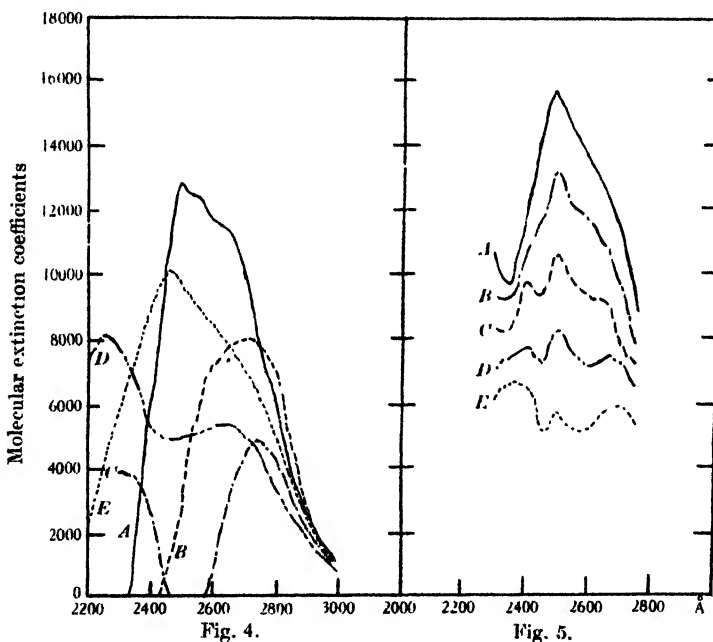


Fig. 4. Curves used in constructing Figs. 7 and 8. A—Absorption of the aminopyrimidine when bound to thiazole. B—Absorption of the oxypyrimidine when bound to thiazole. C—Absorption of free aminopyrimidine. D—Smakula's curve for Windaus's ethoxypyrimidine replotted with molecular extinction coefficients as ordinates. E—Sulphonc acid of the aminopyrimidine. (Curve of Williams *et al.* and Ruehle).

Fig. 5. Hypothetical curves of various stages of dissociation of the aminovitamin. A—10%, B—30%, C—50%, D—70%, E—90%, dissociated.

the Holiday curve gives an approximation to the absorption of the aminopyrimidine as it may be modified by linking with thiazole. It resembles (Fig. 4, A) the absorption of the aminopyrimidinesulphonc acid (Fig. 4, E) with greatly increased extinction values.

Smakula [1934] has given the absorption of a "hydrolysis product" of the vitamin, the analysis of which agrees with the result of deamination of the

vitamin (Windaus formula). His curve may be presumed to represent the summation of the absorptions of the quaternary thiazole and of the oxypyrimidine when bound to the thiazole. Deduction of the absorption of the thiazole methiodide gives the curve of the bound oxypyrimidine (Fig. 4, B).

In obtaining these curves, it has been assumed that in each case the vitamin curves represented the undissociated complex.

There is no assurance that the absorptions of the amino- and oxy-pyrimidines are similar to those of their sulphonic acids. For the free oxypyrimidine we have employed, for reasons stated in the next section, the curve (Fig. 4, D) given by Smakula [1934] for a pyrimidine obtained by Windaus *et al.* [1934] by oxidative cleavage of the vitamin with nitric acid. To obtain the absorption of the free aminopyrimidine (Fig. 4, C), we deducted the absorption of tertiary thiazole from the Holiday curve for the vitamin in neutral alcohol on the assumption that this curve, which has very low extinction values, represents the vitamin after complete dissociation into its aminopyrimidine and thiazole components. The assumption that the pyrimidine has suffered little if any deamination is based upon the observation of Holiday that on acidifying a solution of the vitamin in neutral alcohol, its absorption increased and tended towards the form of curve exhibited when the vitamin is originally dissolved in acid alcohol. As the addition of acid could not introduce an amino-group, it is evident that the pyrimidine in the Holiday neutral alcohol curve could not have been an oxypyrimidine. The addition of acid apparently leads to the recombination of the dissociated components into a quaternary thiazole.

Two series of hypothetical absorption curves of various proportions of free and bound aminopyrimidine and of free and bound oxypyrimidine were prepared. Summations of the absorption due to various ratios of quaternary and tertiary thiazole were then made with the corresponding proportions of free and bound pyrimidines of each type, yielding two families of curves indicative of the effect of dissociation of the pyrimidine-thiazole complex, one for the amino- and the other for the oxy-pyrimidine (Figs. 5 and 6). At each level of dissociation it should be possible to construct a series of curves showing the effect of deamination. In preparing these summation curves, errors are introduced at wave-lengths shorter than about 2400 Å, because the effects of negative absorptions or apparent fluorescences of the bound forms of the pyrimidines have been neglected.

We have compared several vitamin absorption curves with the most similar of these hypothetical curves in Figs. 7 and 8 of which the legends are self-explanatory. Obviously, the Holiday acid alcohol curves, the Holiday neutral alcohol curve and that of the Smakula deamination product are also accounted for, since the hypothetical curves were derived from these. No quantitative significance is to be attached to the figures stated for the extent to which each of the processes has occurred in the cases cited; they are to be regarded merely as roughly indicative of the changes which may have occurred in the very dilute solutions employed for the measurements. In all these comparisons the disagreements are similar in type. The hypothetical curves usually have too little absorption in the short-wave region and too much in the longer. The error is probably chiefly the result of the assumption of too small a contribution from quaternary thiazole. The agreement appears close enough to indicate that the two assumed reactions (reversible dissociation of the quaternary thiazole and deamination of the pyrimidine component) are sufficient to account for the diversity of curves published for the vitamin.

From these considerations it appears that the most nearly correct curves for the absorption of the aminopyrimidine-thiazole compound are those of Peters

and Philpot [1933] or of Holiday [1935] in acid alcohol. When the compound is largely dissociated into its components the absorption assumes the two-banded form obtained by Holiday in neutral alcohol. Subsequent acidification increases the absorption by inducing a recombination. When dissociation is relatively

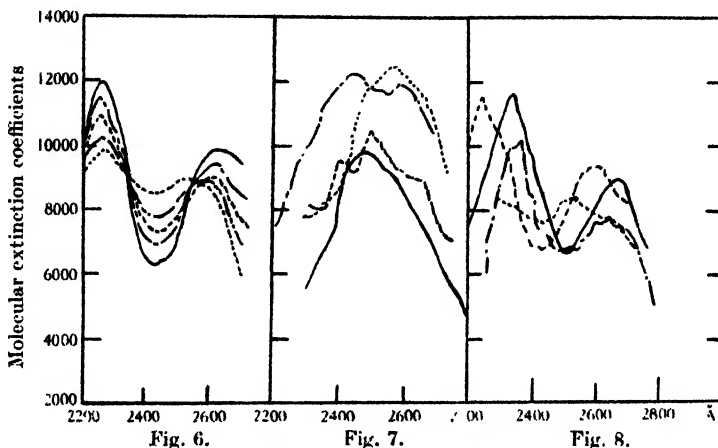


Fig. 6. Hypothetical absorption curves for the deaminated vitamin in various stages of dissociation. — 10%, - - - 30%, . . . 50%, - - - 70%, - - - 90%, dissociated.

Comparisons of observed and hypothetical absorption curves.

Fig. 7. — Vitamin B₁ dissolved in neutral alcohol and re-acidified (curve of Holiday). - - - Hypothetical curve for amino-vitamin 50% dissociated. . . . Vitamin B₁ (curve of Smakula). . . . Hypothetical curve of undissociated vitamin, 50% deaminated.

Fig. 8. . . . Vitamin B₁ curve of Wintersteiner *et al.* - - - Hypothetical curve for the oxy-pyrimidinethiazole complex, 30% dissociated. - - - Vitamin B₁ prepared by Van Veen. - - - Hypothetical curve for vitamin 70% dissociated and 50% deaminated.

slight and accompanied by some deamination, curves of the Smakula [1934] form result. Further dissociation, accompanied probably by variable amounts of deamination, yields curves of the type obtained in neutral alcohol or water by Wintersteiner *et al.* [1935] for their product, and by ourselves for samples obtained from Prof. Peters, from Dr Van Veen and from Profs. Suzuki and Ohdake.

The pyrimidine component and its absorption.

If the free vitamin base corresponding to the hydrochloride is formed in a manner analogous to the thiazole methiodide, the parent pyrimidine according to Williams's formula is 5-hydroxy-6-ethyl-4-aminopyrimidine. The assumption of a hydroxy-group in the parent pyrimidine, rather than the very unlikely chlorine, would resolve the most serious discrepancy between the empirical formulae of Williams's pyrimidine (analysed as sulphonic acid) and the pyrimidine, C₇H₁₀O₂N₂ of Windaus *et al.* [1934]. The presence of but two nitrogen atoms in the latter indicates deamination of the original pyrimidine to have occurred during the action of nitric acid on the vitamin.

Restoration of the amino-group and saponification of the ethoxyl group, which was introduced during the isolation, would make the formula for the original pyrimidine C₆H₇ON₃, which differs from the pyrimidine of the Williams formula only in lacking a methyl group. During the action of nitric acid this might have been removed by oxidation and loss of carbon dioxide.

Esterification of the carboxyl formed by oxidation of the methyl group can be eliminated as it would yield a product containing more oxygen than Windaus found. The hydroxyl introduced by deamination would in the acid solution be expected to undergo a lactim-lactam rearrangement so that it would not be available for replacement by ethoxyl. It is therefore likely that the esterification in Windaus's pyrimidine occurred on the hydroxyl initially present on the carbon atom which becomes linked to thiazole in the vitamin.

The absorption of Windaus's ethoxy-derivative of the oxypyrimidine gives some information as to the allocation of this hydroxyl group. If the ethoxyl occurred in the 2-position, the saponified product would be a methylthymine, and both it and its ethoxyl derivative should exhibit one absorption band [Heyroth and Loofbourow, 1934, 1; Austin, 1934]. As the Windaus product showed two bands, a 2-ethoxyl structure appears unlikely.

The absorption of the free oxypyrimidine cannot be determined from the vitamin curves. When bound to thiazole, its absorption (Fig. 4 B) differs from that of its sulphonic acid in lacking the short-wave band. This is understandable in view of the possibility of a lactam-lactim rearrangement. It was therefore necessary to choose for the curve to represent the free oxypyrimidine in the comparisons of the preceding section between a one-banded and a two-banded curve.

Actually, two sets of the summation curves were prepared, using for the absorption of the free oxypyrimidine as a one-banded curve the absorption of thymine, and as a two-banded curve, that of the Windaus ethoxy-derivative. The curves based upon the former failed to give agreement with the observed vitamin curves. This affords further support for the view that position 2 is not the site of the hydroxyl group in the free pyrimidine, and leaves only positions 5 or 6 available for it. It is interesting to note here that 5-hydroxypyrimidine derivatives, *e.g.* isobarbituric acid, are easily oxidised to dipyrimidines, fluorescent in alkaline solution. This may point to a mechanism for the production of fluorescent compounds on oxidation of the vitamin [Peters, 1935].

SUMMARY.

1. Holiday's observation of variations in the ultra-violet absorption spectrum of vitamin B₁ prepared by Peters has been confirmed.
2. Samples of the vitamin prepared by Seidell, Suzuki and Ohdake, and Van Veen have in aqueous solutions ultra-violet absorptions similar to those reported by Wintersteiner, Williams and Ruchle.
3. A correlation between biological activity and ultra-violet absorption has been demonstrated for three crystalline preparations, but because of the ease with which the absorption is altered must be considered merely as a coincidence.
4. The diversity of ultra-violet absorption spectra reported for the vitamin is attributed to two independent processes occurring to varied extents: (a) reversible dissociation of the vitamin into an aminopyrimidine derivative and a thiazole derivative and (b) deamination of the aminopyrimidine.
5. The curve presented by Peters and Philpot for acid alcohol solutions most nearly represents the vitamin. Holiday's curves in neutral alcohol represent the result of the breaking of the quaternary linkages of the thiazole. Other curves represent intermediate stages, accompanied by some deamination of the pyrimidine.
6. A consideration of the possible structures indicated by the analysis of the pyrimidines isolated from the vitamin by Windaus and by Williams leads to the

conclusion that the pyrimidine component of the vitamin has one amino, one hydroxyl and two methyl (or one ethyl) groups as substituents. The spectrographic evidence suggests that the hydroxyl group is probably not in the 2-position.

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XCI. METABOLISM OF NORMAL AND TUMOUR TISSUE.

XIV. A NOTE ON THE METABOLISM OF MEDULLA OF KIDNEY.

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GYÖRGY *et al.* [1928] showed that medulla of kidney belongs to the most strongly glycolysing tissues. They found for the anaerobic glycolysis of medulla of kidney of the rat values of $Q_{10} = 25.6$ as average and 34.1 as maximum. The aerobic glycolysis of the slices in Ringer solution was found to amount to $Q_{10} = 15$ to 17 . This aerobic glycolysis however was stated by these authors to disappear when serum was used instead of Ringer solution.

In the course of investigations on the glycolysis of animal tissues we found it desirable to reinvestigate these little-known observations. We could confirm them fully with the exception of one important point: we did not find a disappearance of aerobic glycolysis in serum, even in the serum of the same species. As a matter of fact the aerobic glycolysis in serum was exactly the same as in Ringer solution. The respiratory quotient was determined in some instances and found to be about unity, thus confirming the rule that normal tissues with high glycolysing power have a R.Q. of 1.

Medulla of kidney therefore provides in addition to retina another example of normal "resting" tissue with an anaerobic type of metabolism. But in contrast to retina where a great vulnerability of the highly specialised cells may be anticipated, kidney medulla consists of a system of conducting tubules of simple epithelium, where no abnormal susceptibility appears likely.

This is relevant to the theories which regard an anaerobic type of metabolism as associated with growth or even with malignant growth only. We suggest that the cause of an anaerobic type of metabolism is in all such cases merely a disparity between blood supply, *i.e.* oxygen supply, and energy requirements of the tissue *in vivo*.

Methods.

Slices were cut from that part of the kidney only which is known as the pyramid. The metabolism was measured in the Warburg apparatus at 37.5° in the usual way. Serum was inactivated for 2 hours at 56° , glucose was then added to give a concentration of 0.2% . R.Q. was determined by the method of Dickens and Simer [1930].

RESULTS.

Species	Medium	Hours	Q_{O_2}	Q_{CO_2}	$Q_{CO_2}^{N_2}$	R.Q.
Guinea-pig	Bicarb.-glucose-Ringer	1st	- 7.4	14.2	33.6	—
		2nd	- 8.6	13.3	25.6	—
		3rd	- 7.1	12.0	20.8	—
	Bioarb.-glucose-Ringer	1st	- 5.7	15.7	26.7	—
		2nd	- 9.2	17.1	26.7	—
		3rd	- 7.1	9.3	19.7	—
	Horse serum	1st	- 10.0	17.4	28.6	—
		2nd	- 5.4	11.9	23.3	—
	Horse serum	1st	- 7.0	13.5	28.2	—
		2nd	- 6.5	10.8	25.9	—
	Guinea-pig serum	1st	- 9.3	14.0	29.4	—
		2nd	- 9.4	11.6	21.8	—
Cat	Bicarb.-glucose-Ringer	1st	- 2.7	9.5	13.1	—
		2nd	- 1.6	6.6	11.9	—
	Bicarb.-glucose-Ringer	1st	- 0.4	7.5	13.9*	—
Guinea-pig	Phosphate-glucose-Ringer	1st	- 10.3	—	—	} 0.99
		2nd	- 8.7	—	—	
		3rd	- 8.9	—	—	
		4th	- 8.9	—	—	
		5th	- 8.7	—	—	
	Phosphate-glucose-Ringer	1st	- 10.1	—	—	} 0.98
		2nd	- 9.6	—	—	
		3rd	- 9.2	—	—	
		4th	- 9.5	—	—	
		5th	- 9.3	—	—	

* In absence of glucose $Q_{CO_2}^{N_2} = 2.4$ (falling).

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XCIV. METABOLISM OF NORMAL AND TUMOUR TISSUE.

XV. THE RESPIRATORY QUOTIENT OF BRAIN CORTEX.

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ELLIOTT AND BAKER [1935] have repeated a number of observations made by ourselves on respiratory quotients of various isolated tissues [Dickens and Šimer, 1930, 2; 1931, 2; Dickens and Greville, 1933, 1, 2; 1935]. While duplicating many of our results they state that in some cases "important differences from their findings have been observed". On these differences they base a fundamental criticism of our work on metabolism.

The most striking difference reported was in the case of rat-brain cortex, where Elliott and Baker report a mean R.Q. 0.86 (extremes 0.93-0.78). Our results were much higher being in the region of unity, thus supporting the view held by nearly all workers that in brain the oxidation of carbohydrate preponderates [Gayda, 1914; Loebel, 1925; Meyerhof and Lohmann, 1926, 1, 2; Himwich and Nahum, 1929; 1930; Himwich and Fazikas, 1932; Himwich, 1932; Ashford and Holmes, 1931; Gerard and Schachter, 1932; Weil-Malherbe, unpublished].

Despite the fact that their results were in opposition to this prevailing view and that their experiments were based on a single technique for determination of R.Q. and on a comparatively small number of experiments, Elliott and Baker suggest that their own results should be accepted as correct.

In view of the fact that by the use of our three independent methods of R.Q. determination we have never observed values of the order claimed by Elliott and Baker, we now report (Table I) all successfully completed experiments on rat-brain cortex made during the last five years. Fig. 1 shows the graphical representations of these results. They are quite clearly those to be expected on statistical grounds for a tissue of R.Q. 1, using a method subject to an error of a few per cent.¹

Elliott and Baker's complete results on rat-brain cortex are included in Fig. 2: the difference between these results and our own is far outside any possible error of our experiments. It will be noted that whilst the results in our experiments follow the statistical law, those of Elliott and Baker show no peak but rather a wide plateau indicating a lack of uniformity in their results.

Elliott and Baker attempt to explain their low results by a complicated theory based on the action of salt solutions on tissues. They suppose, too, that our experiments lasted for 5 hours which was not the case (see Table I), and they also assume wrongly that the R.Q. rose during this period. A simpler explanation of this supposed increased R.Q. will be suggested later.

¹ Since the R.Q. of medulla [Himwich and Fazikas, 1932] and of peripheral nerve [Meyerhof and Schmidt, 1929] which consist largely of white matter is low, it is possible that a slight contamination with white matter may account for a few results being low.

Table I. *Respiratory quotient of rat-brain cortex; measurements of Dickens and co-workers.*

Date	Exp. no.	Respiratory quotient	Time of exp. (min.)	Medium	Method
3. iv. 30	1	1.00	120	PG	D & S 1
29. iv. 30	2	1.02	180	"	"
	3	1.00	180	"	"
	4	1.03	180	"	"
30. iv. 30	5	0.96	150	"	"
	6	0.98	150	"	"
	7	1.00	120	"	"
7. vi. 30	8	0.94	75	"	"
	9	1.00	105	"	"
8. vi. 30	10	0.96	165	"	"
	11	0.98	135	"	"
10. vi. 30	12	0.98	180	"	"
	13	0.97	180	"	"
11. iii. 31	14	1.00	75	BG	D & S 2
12. iii. 31	15	0.99	90	"	"
13. iv. 31	16	1.00	100	"	"
14. iv. 31	17	0.99	180	"	"
	18	0.99	180	"	"
30. vi. 31	19	0.94	180	"	"
	20	0.96	180	"	"
1. v. 31	21	1.04	180	BG (serum)	"
2. v. 31	22	0.95	180	BG	"
	23	0.95	180	"	"
15. xii. 31	24	0.96	150	BL	"
16. xii. 31	25	1.00	150	"	"
17. xii. 31	26	1.01	180	BG	"
11. i. 32	27	0.97	180	"	"
	28	1.02	180	"	"
13. i. 32	29	0.97	160	BF	"
14. i. 32	30	1.01	210	BL	"
13. iv. 32	31	1.01	160	BF	"
28. ii. 33	32	1.00	200	BG	"
	33	1.01	200	BF	"
2. iii. 33	34	0.91	180	BG	"
	35	0.95	180	BF	"
3. iii. 33	36	1.01	120	PG	"
	37	1.03	120	PF	"
19. vii. 33	38	1.03	90	BG	D & G
24. vii. 33	39	0.99	120	"	"
30. i. 34	40	0.98	120	"	"
3. ii. 35	41	1.02	90	PG	D & S 1
	42	1.01	90	"	"
5. ii. 35	43	1.03	120	BG	D & S 2
25. iv. 35	44	1.00	80	PG	D & S 1
	45	1.02	80	"	"
4. vi. 35	46	0.96	180	"	"
5. vi. 35	47	1.02	120	"	"
	48	1.01	120	"	"
3. v. 34	49	0.99	175	BG	D & S 2
4. ix. 34	50	0.96	180	"	"
23. xi. 34	51	0.97	150	"	"
26. xi. 34	52	0.92	180	"	"
17. xi. 33	53	0.96	185	BL	"
26. xi. 33	54	0.97	165	"	"

Abbreviations. Medium: B = bicarbonate saline

P = phosphate saline

G = medium contained 0.2% glucose

F = medium contained 0.2% fructose

L = medium contained 0.2% lactate

Methods: D & S 1 = Dickens and Šimer (1930, 1)

D & S 2 = Dickens and Šimer (1931, 1)

D & G = Dickens & Greville (1933, 2)

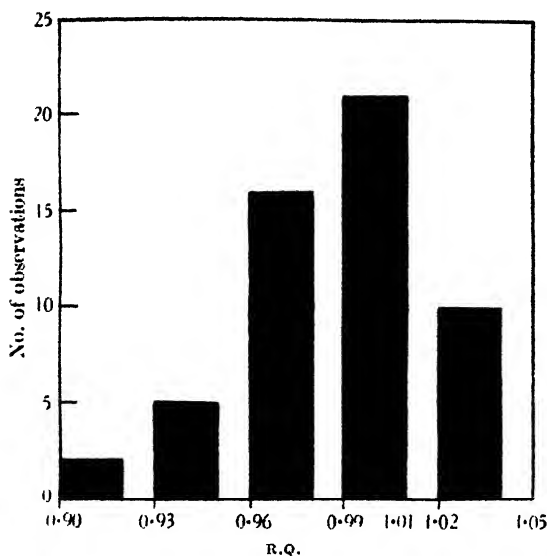


Fig. 1. Respiratory quotient of rat-brain cortex [Dickens and co-workers].

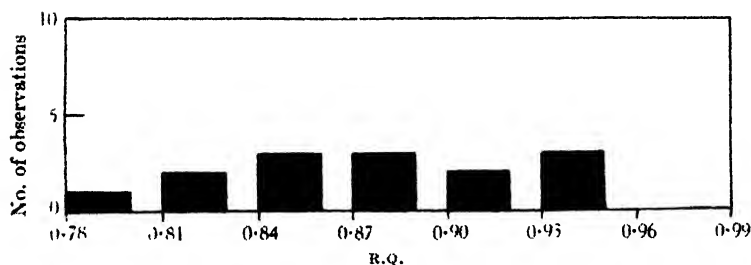


Fig. 2. Respiratory quotient of rat-brain cortex [Elliott and Baker, 1935].

Table II. *Rat-brain cortex: results of Elliott and Baker [1935], Table II, p. 2434.*

Exp. no.*	Rate of respiration (μ l. O ₂ per hour)	Respiratory quotient
15	150	1.00
14	160	0.93
7	182	0.93
4	183	0.93
8	213	0.90
3	217	0.91
1	272	0.81
12	300	0.83
2	302	0.88
13	316	0.89
5	328	0.86
11	333	0.85
10	342	0.84
6	366	0.78
9	380	0.87

* These numbers refer to the 15 individual estimations of Elliott and Baker, Table II, samples 1 to 10; numbering from the top downwards.

It appeared to us probable that there was some unsuspected error in the technique used by Elliott and Baker.

Inspection of their results [Elliott and Baker, 1935, Table II] revealed a possible source of such an error. The quantities of tissues used by Elliott and Baker are very large and consequently the rate of gas exchange is much higher than in our experiments. In Table II Elliott and Baker's results for rat-brain cortex are recorded in order of increasing gas exchange calculated from their figures for respiration and tissue weight.

It is evident from inspection of this table that there is a general fall in the observed "respiratory quotient" with increasing rate of gas exchange. In six experiments out of fifteen where the gas exchange is below 220 $\mu\text{l. O}_2$ per hour, the "R.Q." is never below 0.90 and the average is 0.93. In the remaining nine observations where the gas exchange exceeds 250 $\mu\text{l.}$ per hour there is not one "R.Q." of 0.90 or above and the average is 0.845.

The one value of R.Q. 1.0 in Elliott and Baker's table is explained by them as being due to the prolonged action of salt solution. It seems to us more probable that the correct explanation is that this was the only experiment in which adequate gas equilibrium occurred, the gas exchange being in this particular experiment at the more reasonable level of 150 $\mu\text{l. O}_2$ per hour.

The need for caution in this respect was emphasised in our earlier papers [Dickens and Šimer, 1931, 1, 2] where we pointed out that with our method gas exchanges above a certain magnitude were liable to introduce serious error. It is of course impossible for us to say what limit of gas exchange is permissible for the particular apparatus and rate of shaking used by Elliott and Baker, but the critical survey of their results suggests that it may be of the same order as in our vessels, *viz.* 100–150 $\mu\text{l.}$ per hour. The errors introduced by ignoring this precaution are not susceptible to quantitative treatment. They could perhaps account also for some of Elliott and Baker's results on highly glycolysing tissues, where it appears probable that the gas exchange due to very large glycolysis might easily lead to quite indeterminate irregularities in these results.

To my former and present colleagues, Dr F. Šimer, Mr G. D. Greville and Dr H. Weil-Malherbe, I would like to express my thanks for many of the measurements included in Table I.

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XCV. STUDIES ON BRAIN METABOLISM.

I. THE METABOLISM OF GLUTAMIC ACID IN BRAIN.

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It has long been realised that glutamic acid differs from most amino-acids, since it is oxidised in organs whose metabolism is supposed to be mainly concerned with carbohydrate and which are quite inert towards most of the other amino-acids. This suggests a connection between glutamic acid and carbohydrate metabolism.

Thunberg [1920] first showed that glutamic acid was the only amino-acid in presence of which washed frog muscle decolorised methylene blue. Harrison [1925] described aerobic oxidation of glutamic acid by washed frog muscle. Needham [1930] showed that glutamic acid is oxidised to succinic acid by the muscle of ox, rabbit, pigeon and frog. Holmberg [1934] prepared extracts from washed muscle which reduced methylene blue in presence of glutamic acid.

Thunberg [1923] demonstrated oxidation of glutamic acid by minced peripheral nerve and Quastel and Wheatley [1932] by brain. Krebs [1935, 1] observed increased respiration of brain and retina in the presence of glutamic acid.

The probable oxidation of glutamic acid by tumour tissue was indicated by the observation of Fleisch [1924] that a preparation of washed, minced Jensen rat sarcoma reduces methylene blue in presence of glutamic acid and by unpublished experiments of Dickens and Weil-Malherbe.

METHODS.

Respiration was measured by the manometric method of Warburg [1926]. Unless otherwise stated, brain slices (grey matter only) of rats or guinea-pigs were used. The tissue (10–15 mg. dry wt.) was suspended either in phosphate saline [Krebs, 1933] or in bicarbonate saline [Krebs and Henseleit, 1932]. When phosphate saline was used, the manometer was filled with oxygen and the respiratory CO_2 was absorbed by 0.2 ml. of 10% NaOH in the inner cup of the vessel. For the experiments with bicarbonate saline a gas mixture containing 5% CO_2 was used. Any substrate added was neutralised to litmus paper. All experiments were done at 37.5°.

Ammonia and glutamine were determined according to Krebs [1935, 1, 2] with the apparatus of Parnas and Heller.

Units. The amount of metabolites formed or disappearing is expressed in $\mu\text{l.}$ (1 millimol = 22400 $\mu\text{l.}$) or in Q -values $\left(\frac{\mu\text{l.}}{\text{mg. dry weight} \times \text{hours}} \right)$ with a corresponding index.

Nomenclature. The nomenclature of the amino-acids is that of Freudenberg and Karrer [cf. Krebs, 1935, 1].

Further experimental details will be given in the following sections.

I. *l(+)-Glutamic acid as a substrate of brain respiration.*

l(+) Glutamic acid maintains the respiration of brain slices which without the addition of a suitable substrate falls off rapidly. Glutamic acid is in this respect equal or even superior to glucose (Table I, see also Krebs, 1935, 1).

Table I. *Respiration of brain slices in presence of l(+)-glutamic acid.*
Phosphate saline.

Species	Substrate added	Q_{O_2}		
		1st	2nd	3rd hr.
Rat	0	- 8.5	- 4.8	- 1.9
	0.2% glucose	- 9.5	- 9.5	- 10.0
	<i>M</i> /100 <i>l(+)</i> -glutamic acid	- 14.4	- 12.0	- 10.4
Guinea-pig	0	- 6.3	- 3.4	- 1.7
	0.2% glucose	- 8.8	- 8.7	- 9.4
	<i>M</i> /50 <i>l(+)</i> -glutamic acid	- 12.8	- 11.5	- 10.6
Guinea-pig	<i>M</i> /100 <i>l(+)</i> -glutamic acid	- 14.5	- 11.2	- 9.1
	<i>M</i> /1000 <i>l(+)</i> -glutamic acid	- 13.9	- 8.9	- 4.8
	<i>M</i> /4000 <i>l(+)</i> -glutamic acid	- 13.4	- 6.3	- 3.7
	0	- 10.3	- 5.7	- 3.6

If the concentration of *l(+)*-glutamic acid is diminished, respiration starts at a high rate and begins to fall after some time indicating exhaustion of the substrate (Table I, last exp.).

An increase of bicarbonate is observed when brain slices are incubated with *l(+)*-glutamic acid (Table II). This indicates disappearance of acid in the course of glutamic acid oxidation.

Table II. *Acid disappearance during the oxidation of l(+)-glutamic acid.*
Brain slices of guinea-pig in bicarbonate saline.

Method	Conc. of <i>l(+)</i> -glu- tamic acid	Q_{O_2}			Q_{O_2}		
		1st	2nd	3rd	1st	2nd	3rd hr.
Warburg [1924], cf. Meyerhof and Lohmann [1926]	0	- 2.0	- 0.4	- 0.1	- 5.7	- 3.4	- 2.2
	<i>M</i> /50	- 4.9	- 3.8	- 3.6	- 10.1	- 9.2	- 5.6
Dickens and Šimer [1931]	0	-	- 0.86	-	-	- 3.5	-
	<i>M</i> /50	-	- 2.9	-	-	- 14.3	-

The R.Q. is little below unity (Table III). The theoretical value for complete oxidation is 1.1.

Table III. *R.Q. of brain slices in presence of l(+)-glutamic acid.*

Method	Species	Medium	Substrate added	Time of exp. hrs.	Q_{O_2}	R.Q.	Remarks
Dickens and Šimer [1930]	Rat	Phosphate saline	<i>M</i> /100 <i>l(+)</i> -glutamic acid	2	- 10.7	0.91	
			0.2% glucose		- 10.8	1.02	
			0		- 6.6	1.01	
Dickens and Šimer [1930]	Guinea- pig	Phosphate saline	<i>M</i> /100 <i>l(+)</i> -glutamic acid	2	{ - 12.3 - 12.4	{ 0.95 0.97	Dupli- cate experi- ments
			0.2% glucose		{ - 9.5 - 9.9	{ 0.97 0.98	
Dickens and Šimer [1931]	Guinea- pig	Bicar- b. saline	<i>M</i> /100 <i>l(+)</i> -glutamic acid	3	- 14.3	0.91	
			0		- 3.5	1.07	

In presence of other oxidisable substrates *l*(+)-glutamic acid raises the respiration still further (Table IV). This indicates a summation of oxidations. Brain thus differs from kidney or yeast, in which, in the presence of several substrates, there is competition for the available oxygen [Krebs, 1935, 1].

Table IV. *Summation of oxidations in brain. Phosphate saline.*

Species	Substrate added	Q_{O_2}		
		1st	2nd	3rd hr.
Guinea-pig	0.2% glucose	- 9.9	—	—
	<i>M</i> /50 <i>l</i> (-)-glutamic acid	- 11.3	—	—
	0.2% glucose + <i>M</i> /50 <i>l</i> (+)-glutamic acid	14.6	—	—
Rat	<i>M</i> /100 <i>l</i> (+)-glutamic acid	- 14.3	- 10.2	- 7.1
	<i>M</i> /100 lactic acid	- 17.1	- 15.3	- 12.9
	<i>M</i> /100 lactic acid + <i>M</i> /100 <i>l</i> (+)-glutamic acid	- 21.5	- 19.2	- 15.9
	<i>M</i> /100 pyruvic acid	- 21.1	- 18.2	- 16.6
	<i>M</i> /100 pyruvic acid + <i>M</i> /100 <i>l</i> (+)-glutamic acid	24.4	- 21.5	- 17.5
Guinea-pig	<i>M</i> /100 <i>l</i> (+)-glutamic acid	11.5	- 10.6	—
	<i>M</i> /100 succinic acid	- 10.9	- 4.6	—
	<i>M</i> /100 <i>l</i> (-)-glutamic acid + <i>M</i> /100 succinic acid	- 18.6	15.8	—

II. Other amino-acids.

l(+)-Glutamic acid is the only amino-acid oxidised in brain. The following 12 naturally occurring amino-acids have been tested and found ineffective in maintaining brain respiration: *l*(+)-alanine, *l*(+)-valine, *l*(-)-leucine, *l*(-)-methionine, *l*(-)-proline, *l*(-)-hydroxyproline, *dl*-serine, *l*(-)-aspartic acid, *l*(+)-ornithine, *l*(+)-arginine, *l*(-)-histidine and *l*(-)-tryptophan (concentration *M*/100 in all experiments).

III. Glutamine and *d*(-)-glutamic acid.¹

Glutamine like *l*(+)-glutamic acid maintains brain respiration, although not quite so effectively (Table V). The oxidation of glutamine may proceed partially or totally *via l*(+)-glutamic acid, since Krebs [1935, 2] has shown the presence in brain of an enzyme capable of splitting glutamine to *l*(+)-glutamic acid and ammonia.

d(-)-Glutamic acid, the non-natural isomeride, is not oxidised by brain slices. There is even a slight depression of respiration in absence as well as in presence of glucose (Table V and Fig. 1).

The same specificity in the behaviour of brain slices towards the optical isomerides is observed in methylene blue experiments.

The experiments were done in ordinary Warburg manometers. The slices were previously shaken in oxygen long enough to exhaust their stores of preformed substrates and were transferred to the vessels containing bicarbonate saline in the main part and 0.2 ml. of a freshly prepared 2*N* solution of chromous chloride in the inner cup. The gas space was filled with $N_2 + 5\%$ CO_2 . After 10 min. for absorption of the last traces of oxygen the methylene blue was tipped from the side bulb and the time necessary for complete decoloration was noted. After the experiment the slices were dried and weighed. In order to facilitate the comparison with the respiratory experiments the results are expressed in values of Q_{MB} , i.e. μ l. of reduced methylene blue (373.5 mg. = 22400 μ l.) per hour and mg. dry weight.

In view of the fact that 2 mols. of methylene blue correspond to 1 mol. of oxygen, it will be noted from Table VI that the anaerobic oxidation with

¹ I am indebted to Dr H. A. Krebs for samples of both substances.

methylene blue is about 15–20 times smaller than the oxidation in oxygen, a fact which has already been pointed out and discussed by Fleisch [1924]. The experiments however show clearly that *l*(+)-glutamic acid accelerates the decoloration of methylene blue whereas *d*(-)-glutamic acid is slightly inhibiting.

Table V. *Respiration of brain slices in presence of l(+)-glutamic acid, d(-)-glutamic acid and glutamine. Phosphate saline.*

Species	Substrate added	Q_{O_2}		
		1st	2nd	3rd hr.
Rat	0	8.5	4.8	1.9
	<i>M</i> /100 <i>l</i> (+)-glutamic acid	14.4	12.0	10.4
	<i>M</i> /100 glutamine	10.2	8.7	7.0
	<i>M</i> /100 <i>d</i> (-)-glutamic acid	4.6	2.5	1.6
Guinea-pig	0.2% glucose	9.5	9.3	
	0.2% glucose + <i>M</i> /50 <i>d</i> (-)-glutamic acid	8.3	5.5	

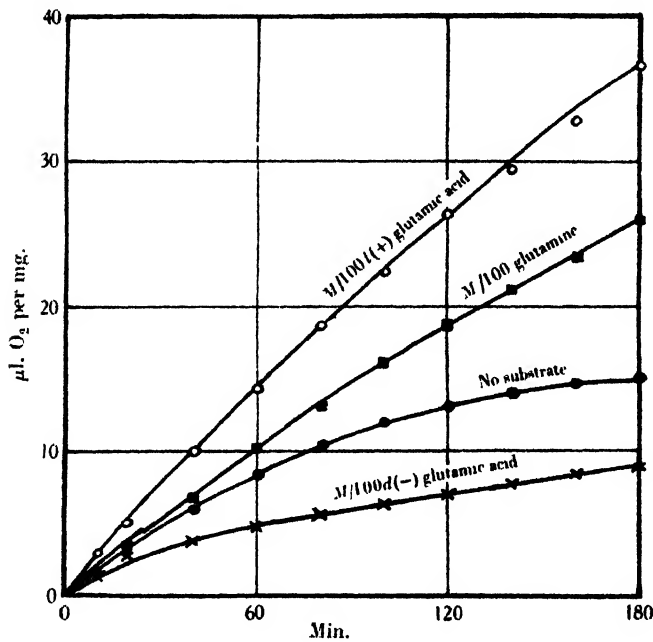


Fig. 1. *Respiration of rat brain in presence of l(+)-glutamic acid, d(-)-glutamic acid and glutamine.*

Table VI. *Reduction of methylene blue by slices of guinea-pig brain in presence of d- and l-glutamic acid. 0.2 mg. of methylene blue in each vessel.*

Substrate added	Dry weight of slices mg.	Deroloration time min.	Q_{MB}	Remarks
0	23.21	20	1.55	Slices previously shaken for 1 hour in O_2
<i>M</i> /50 <i>l</i> (+)-glutamic acid	26.06	12.5	2.21	
<i>M</i> /50 <i>d</i> (-)-glutamic acid	26.17	18	1.53	
0	19.36	49	0.76	Slices previously shaken for 2 hours in O_2
<i>M</i> /50 <i>l</i> (+)-glutamic acid	18.67	34	1.14	
<i>M</i> /50 <i>d</i> (-)-glutamic acid	21.62	48	0.69	

IV. *Glutamic acid deaminase*

Since brain slices only attack the natural isomeride it is the more surprising that extracts of brain not only attack the non-natural *d*(-)-glutamic acid, but even attack it preferentially. *l*(+)-Glutamic acid is also oxidised but more slowly and, relatively to the *d*-acid, the more slowly the more the extract is diluted. This behaviour is shown under aerobic (Table VII) as well as anaerobic conditions (Table VIII).

Table VII. *Oxygen uptake of an extract of fresh ox brain.*

50 g. of fresh ox brain (grey matter) are ground with 25 ml. water and centrifuged. The supernatant creamy emulsion is decanted and diluted with *M*, 100 veronal buffer of p_H 8.2. Each vessel contains 2 ml. of the extract.

Parts of buffer added to 1 part of extract	Substrate added	μ l. O_2 uptake (60 min.)
1	0	103
	<i>M</i> 50 <i>l</i> (+)-glutamic acid	114
	<i>M</i> 50 <i>d</i> (-)-glutamic acid	126
3	0	37
	<i>M</i> 50 <i>l</i> (+)-glutamic acid	46.5
	<i>M</i> 50 <i>d</i> (-)-glutamic acid	55
7	0	15.5
	<i>M</i> 50 <i>l</i> (+)-glutamic acid	22
	<i>M</i> 50 <i>d</i> (-)-glutamic acid	29.5

Table VIII. *Reduction of methylene blue by extracts of fresh brain.*
Thunberg technique.

Extract 1 was identical with that used in the experiment of Table VII, diluted with 10 parts of veronal buffer p_H 8.2. Extract 2 was prepared by grinding a rat brain with 10 parts of *M*, 50 phosphate buffer (p_H 7.4) and centrifuging. Each tube contained 2 ml. of extract and 0.2 ml. of methylene blue solution 1 : 5000.

Extract (no.)	Substrate added	Decoloration time (mm.)
1	0	45
	<i>M</i> 50 <i>l</i> (+)-glutamic acid	33
	<i>M</i> 50 <i>d</i> (-)-glutamic acid	13
2	0	31
	<i>M</i> 50 <i>l</i> (+)-glutamic acid	20
	<i>M</i> 50 <i>d</i> (-)-glutamic acid	9

Unless it is assumed that an enzyme which was previously masked suddenly appears on extraction, one must conclude that on extraction the same enzyme undergoes a change of its specificity towards the two optical isomerides. Change of optical specificity is a well known fact in enzyme chemistry [*cf.* Rona and Ammon, 1933]. In this connection it is of special interest that steric selectivity may change with different methods of preparation [Bamann and Laeverenz, 1930] or with a different position of the catalytically active group on the colloidal carrier [Schwab *et al.*, 1933]. Bamann and Laeverenz [1930] conclude "that the difference of optical selectivity cannot be explained by differences of the active chemical group of the esterases, but is due to a changed association with specific carriers, brought about in the organism".

The behaviour of other organs towards optically isomeric amino-acids differs from that of brain. From the work of Krebs [1933; 1935, 1] it is known that kidney slices oxidise not only the natural, but also the non-natural amino-acids,

the latter even faster in many cases. As to the glutamic acids I find that the *d*-acid is oxidised by kidney slices little less rapidly than the *l*-acid in phosphate saline or in dialysed horse serum (Table IX).

Table IX. *Oxidation of l(+)- and d(-)-glutamic acid by kidney slices (guinea-pig), (1) in phosphate saline, (2) in dialysed horse serum.*

Preparation of the dialysed horse serum: 100 ml. of horse serum were saturated with CO₂ and dialysed in a collodion sac against 2 l. of distilled water saturated with CO₂ [Van Slyke *et al.*, 1923] for 2 days. The water was changed twice daily. The serum was then freed *in vacuo* from dissolved CO₂, neutralised with NaOH to *p*_H 7.4 (phenol red), made up with salts and phosphate buffer to correspond to the phosphate saline and finally inactivated at 56° for 2 hours.

Substrate added		Phosphate saline			Dialysed horse serum		
		Q _{O₂}	Q _{NH₃}	Q _{Amide N}	Q _{O₂}	Q _{NH₃}	Q _{Amide N}
0	1st hr.	- 12.7	} 1.03	1.07	13.4	} 0.86	0.94
	2nd hr.	- 9.7			11.6		
<i>M</i> /50 <i>l</i> (+)-glutamic acid	1st hr.	- 29.1	} 0.63	6.58	29.8	} 0	5.00
	2nd hr.	30.2			31.6		
<i>M</i> /50 <i>d</i> (-)-glutamic acid	1st hr.	- 23.1	} 1.34	1.22	22.5	} 2.03	1.94
	2nd hr.	- 16.4			- 17.4		

Considerable amounts of the amino-acid deaminase pass into solution when kidney slices are incubated in saline [Krebs, 1933] and it is very probable that it is the dissolved part of the enzyme which accounts for the oxidation of the *d*-acids. When the cell has been killed with octyl alcohol oxidation of the *l*-acids is stopped, but that of the *d*-acids continues [Krebs, 1935. 1].

Dickens and Weil-Malherbe [1935] showed that tumour slices attack *l*- and *d*-glutamic acid with about the same velocity. Tumour in this respect behaves like kidney. On the other hand glutamic acid seems to be the only amino-acid oxidised by tumour.

Another difference between brain and kidney is important: whereas a fresh extract of brain still possesses some activity towards *l*(+)-glutamic acid, an extract of kidney is entirely inactive towards all *l*-amino-acids.

These discrepancies were thought possibly to be linked with the difference of the chemical composition of the two tissues, especially with the abundance of lipoids in brain. The assumption of a dissociable complex of the enzyme with some lipoid could explain why brain slices hold the enzyme more strongly than

Table X. *Extract of dry brain powder after acetone extraction. Thunberg technique.*

100 g. of fresh ox brain are treated with 500 ml. of acetone and dried *in vacuo* over P₂O₅. The finely ground dry powder is repeatedly treated at room temperature with 100 ml. of acetone.

Extract 1: 1 part of the powder extracted with 10 parts of *M*/100 veronal buffer (*p*_H 8.2) and centrifuged.

Extract 2: extract 1 diluted 5 times with veronal buffer.

Each tube contained 2 ml. of extract and 0.2 ml. of methylene blue solution 1:5000.

Extract (no.)	Substrate added	Decoloration time (min.)
1	0	33
	<i>M</i> /50 <i>l</i> (+)-glutamic acid	18
	<i>M</i> /50 <i>d</i> (-)-glutamic acid	22
2	0	78
	<i>M</i> /50 <i>l</i> (+)-glutamic acid	81
	<i>M</i> /50 <i>d</i> (-)-glutamic acid	51

kidney or tumour slices, which are comparatively poor in lipoids. The oxidation of the natural amino-acids might then be ascribed to the enzyme-lipoid complex as it exists within the cell and as it persists to a certain extent in the extract of brain, which is rich in lipoids.

The following facts support this view: a concentrated aqueous extract of an acetone-dried brain powder, rich in lipoids, attacks both glutamic acids with about the same velocity; the *l*-acid is even slightly ahead of the *d*-acid. When the same extract is diluted 5 times with water, the activity towards the *l*-acid is completely lost and the *d*-acid only is attacked (Table X).

When the same acetone-dried powder is extracted with ether, even a concentrated aqueous extract will only attack the *d*-acid, but not the *l*-acid. This behaviour is seen both in aerobic and anaerobic experiments. Table XI reproduces an oxygen uptake experiment.

Table XI. *Oxygen uptake with extract of dry brain powder after ether extraction.*

Two different preparations are used in these experiments. Powder 1 is the same as in the previous exp. (Table X) after repeated extraction with large quantities of ether at room temp. Powder 2 was prepared from another ox brain in the same way but was treated with chloroform after ether extraction. Both powders were extracted with 15 parts of *M*/100 veronal buffer (p_H 8.2) and centrifuged. Each vessel contained 2 ml. of extract.

Powder (no.)	Substrate added	Time (hours)	O ₂ -uptake (μl.)
1	0	6	54
	<i>M</i> /50 <i>l</i> (+) -glutamic acid		55
	<i>M</i> /50 <i>d</i> (-) -glutamic acid		80.5
2	0	4	20.5
	<i>M</i> /50 <i>l</i> (-) -glutamic acid		16
	<i>M</i> /50 <i>d</i> (-) -glutamic acid		44

Since the lipid carrier responsible for the steric selectivity of the enzyme is very readily extractable with ether, it probably belongs to the monoamino-phosphatide fraction.

Attempts to restore the power of attacking *l*-amino-acids to kidney extracts by adding emulsions of brain lipoids failed. It therefore seems that the dissociation of the enzyme-lipoid complex is irreversible.

Enzyme-lipoid complexes have repeatedly been reported [cf. Przylecki, 1935]. Ro [1931] and Truszkowski [1934] found that uricase is bound to lipoids in the tissue.

The findings of Abderhalden and Tetzner [1935] that rats fed with *dl*-alanine excrete *d*(-) -alanine unchanged, agree well with the view that in the intact cell only the natural amino-acid is metabolised.

Some properties of the glutamic acid deaminase of brain.

1. *Oxygen uptake.* The oxygen uptake of an extract of the dry brain powder after exhaustive extraction with ether and alcohol or chloroform is small but constant for many hours.

2. *Ammonia production.* Ammonia estimations in the enzyme solution are impaired by the presence of a substance which causes a precipitation in the distillate on addition of Nessler's reagent. This difficulty could be overcome by repeated extraction of the brain powder with alcohol and by adding Nessler's reagent shortly before the colorimetric measurements were carried out. Table XII reproduces such an experiment. It will be noted that the ratio oxygen uptake:

ammonia formation is 1 : 1 and not, as would theoretically be expected, 1 : 2. Krebs [1935, 1], in certain cases, came across the same phenomenon which must be explained by the assumption of a coupled oxidation. Keilin and Hartree [1936] have recently shown that alcohols may serve as substrates for coupled oxidation in the presence of the deaminase-amino-acid system. Since our brain powder has been treated with alcohol, it might be suggested that the coupled oxidation observed was caused by retained alcohol. But the alcohol had been completely removed *in vacuo* over P_2O_5 and it is therefore probable that the powder contained other substances serving as substrates for coupled oxidation.

3. *Specificity of the glutamic acid deaminase.* Table XII shows that other amino-acids of the *d*-series are not oxidised by the enzyme.

Table XII. *Oxygen uptake and ammonia formation with extract of dry brain powder after ether and alcohol extraction.*

Powder 1 of Table XI repeatedly treated with abs. alcohol at room temp. Alcohol removed *in vacuo* over P_2O_5 . Extracted with 15 parts of *M*/100 veronal buffer (p_H 8.2) and centrifuged. Each vessel contained 2 ml. of the extract. Duration of the experiment: 6 hours.

Substrate added	O ₂ uptake μl.	Extra O ₂ μl.	NH ₃ formation μl.	Extra NH ₃ μl.	Ratio O ₂ : NH ₃
0	33.5	--	48.9	--	
<i>M</i> /50 <i>l</i> (+) -alanine	34	+ 0.5	46.6	2.3	-
<i>M</i> /50 <i>dl</i> -alanine	32	- 1.5	48.3	0.6	
<i>M</i> /50 <i>dl</i> -valine	34	+ 0.5	50.6	1.7	
<i>M</i> /50 <i>l</i> (+) -glutamic acid	37	+ 3.5	45.4	3.5	
<i>M</i> /50 <i>d</i> (-) -glutamic acid	38	+ 24.5	73.6	24.7	1 : 1.01

V. Isolation of α -ketoglutaric acid.

The first product of the oxidation of *l*(+) -glutamic acid in brain is α -ketoglutaric acid. It was isolated as 2:4-dinitrophenylhydrazone. This was possible by checking its further oxidation with arsenic [Krebs, 1933]. α -Ketoglutaric acid could only be found in the presence of both *l*(+) -glutamic acid and arsenic. The controls with either arsenic or *l*(+) -glutamic acid alone were negative. Minced brain was used for these experiments instead of slices.

300 g. of fresh ox brain obtained from the slaughter house were minced, suspended in 300 ml. bicarbonate saline, containing *M*/25 *l*(+) -glutamic acid and *M*/1000 As_2O_3 and shaken in 3 wash-bottles with ground stoppers for 4 hours. The wash-bottles were filled with oxygen containing 5% CO_2 . After incubation the contents of the bottles were centrifuged and the proteins in the supernatant fluids were removed by adding 1/10 vol. of 30% trichloroacetic acid. The filtrate was acidified with 1/5 vol. of conc. HCl and 100 ml. of 1% sol. of 2:4-dinitrophenylhydrazine in 2*N* HCl was added. After 12 hours in the ice chest the precipitate was filtered off, redissolved in 2*N* Na_2CO_3 and reprecipitated with 2*N* HCl. The precipitate was washed at the centrifuge with water, dried and recrystallised once from ethyl acetate—light petroleum and once from ethyl acetate. 123 mg. of a lemon-yellow crystalline product were obtained; m.p. 224° (uncorr.); m.p. of a sample prepared from pure α -ketoglutaric acid 224° (uncorr.); mixed m.p. 224° (uncorr.).

(Found: C, 40.26; H, 3.35; N, 17.12%. Calc.: C, 40.44; H, 3.09; N, 17.18%.)

The crude precipitate contained considerable amounts of the 2:4-dinitrophenylhydrazone of a second keto-acid which was encountered also in absence of either arsenic or glutamic acid. It was red, amorphous and easily soluble in Na_2CO_3 with a dark brown colour. With alcoholic KOH it gave a deep violet colour. Its properties agree in many respects with the bis-2:4-dinitrophenylhydrazone of glyoxylpropionic acid which has been described by Veibel [1931]

and Mayer [1931]. Yet the substance does not seem to be identical with this compound. Similar or identical 2:4-dinitrophenylhydrazones from brain have been described by Kraut and Nefflen [1935] and Johnson [1936].

Attempts to use semicarbazide for the fixation of α -ketoglutaric acid, following Hahn *et al.* [1929], were entirely unsuccessful. Semicarbazide in the concentrations used by Hahn did not even depress the oxygen uptake of brain slices in presence of α -ketoglutaric acid to any considerable extent. This experience makes the usefulness of this reagent for the accumulation of keto-acids under the experimental conditions described very doubtful.

VI. The fate of the ammonia derived from deamination of *l*(+)-glutamic acid.

Krebs [1935, 2] has shown that kidney and brain slices synthesise glutamine from *l*(+)-glutamic acid and ammonia. It is therefore not surprising that no free NH_3 is found when *l*(+)-glutamic acid is oxidised by brain slices, since all the NH_3 liberated reacts at once with excess glutamic acid to form glutamine. Krebs found however no increase of the total NH_3 (free NH_3 + amide-N) as compared with the control experiment, when glutamic acid was present. Only a shift in the relation: amide-N/free NH_3 was observed, the amide-N content being increased and the free NH_3 disappearing in the presence of glutamic acid. But the sum of both fractions was substantially the same as in the control. The same puzzling phenomenon was encountered by Needham [1930] in muscle where glutamic acid is oxidised to succinic acid without increased formation of either ammonia or amides.

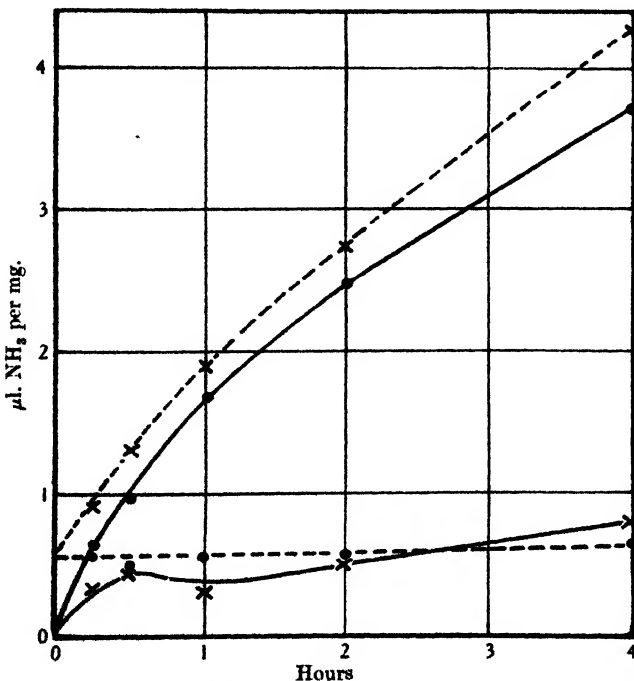


Fig. 2. Ammonia and glutamine formation by slices of guinea-pig brain.

Full line: free ammonia.

× — × *M*/50 *l*(+)-glutamic acid.

Dotted line: amide-nitrogen.

• — • without substrate.

Table XIII. *Ammonia and glutamine formation by slices of guinea-pig brain without added substrate and in presence of M/50 l(+)-glutamic acid. Phosphate saline.*

Time min.	No substrate			l(+)-Glutamic acid		
	$\mu\text{l. NH}_3$	$\mu\text{l. Amide-N}$	$\mu\text{l. total NH}_3^*$	$\mu\text{l. NH}_3$	$\mu\text{l. Amide-N}$	$\mu\text{l. total NH}_3^*$
	per mg. tissue (dry weight)					
15	0.63	0.59	1.22	0.33	0.90	1.23
30	0.96	0.49	1.45	0.44	1.30	1.74
60	1.68	0.55	2.23	0.27	1.90	2.17
120	2.47	0.54	3.01	0.51	2.75	3.26
240	3.70	0.65	4.35	0.80	4.25	5.05

* Total NH_3 sum of free NH_3 and amide-N.

Our own experiments have confirmed Krebs's findings. The increase of amide-N in the presence of l(+)-glutamic acid is accompanied in the control experiment by an increase of free NH_3 which is only slightly smaller, so that practically all the NH_3 derived from the deamination of glutamic acid disappears. Fig. 2 shows a time curve of the NH_3 and glutamine formation in presence and in absence of glutamic acid.

The fate of the NH_3 derived from deamination of glutamic acid will be discussed fully in a subsequent paper dealing with the problem of formation and metabolism of NH_3 in brain.

VII. Reversibility of the reaction l(+)-glutamic acid \rightarrow α -ketoglutaric acid.

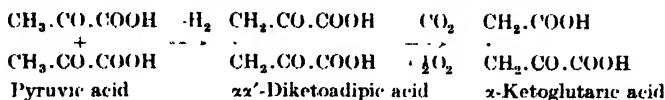
An increased amount of amide-N is found in the presence of glucose, pyruvic acid and α -ketoglutaric acid. Addition of NH_3 further enhances the formation of amide-N. Simultaneously NH_3 disappears from the solution, the largest dis-

Table XIV. *Ammonia-binding mechanism and glutamine formation. Guinea-pig brain.*

Substrate added	mg. tissue (dry weight)	Incubation (hours)	Medium	$\mu\text{l. NH}_3$		Q_{NH_3}	$Q_{\text{Amide-N}}$
				Initial	Final		
0	18.27	3	Phosphate	0	77.0	1.40	0.24
M/50 α -ketoglutaric acid	18.28		saline	0	27.4	0.50	0.42
0	15.81	2	Bicarbonate	0	39.2	1.24	0.30
0.2% glucose	15.09		saline	0	6.2	0.20	0.57
M/50 α -ketoglutaric acid	16.83			0	16.3	0.48	0.52
M/50 α -ketoglutaric acid + 0.2% glucose	16.17			0	0	0	0.46
0	15.64			85	102	0.54	0.31
0.2% glucose	18.26			85	41.3	-1.20	0.84
M/50 α -ketoglutaric acid	14.04			85	77.3	-0.27	0.55
(M/50 α -ketoglutaric acid + 0.2% glucose	12.21			85	29.7	-2.26	1.22
0	17.67	2	Bicarbonate	0	47.2	1.34	0.41
0.2% glucose	18.85		saline	0	10.6	0.28	0.52
M/50 pyruvic acid	14.50			0	8.7	0.30	0.45
M/50 pyruvic acid + 0.2% glucose	16.94			0	0	0	0.40
0	14.54			91.5	119	0.95	0.33
0.2% glucose	20.97			91.5	40.8	-1.21	0.82
M/50 pyruvic acid	17.64			91.5	80.5	-0.31	0.61
(M/50 pyruvic acid + 0.2% glucose	10.09			91.5	57	-1.71	1.23
0	14.18	2	Bicarbonate	91	113	0.78	0.30
0.2% glucose	14.65		saline	91	54	-1.26	0.78
M/50 pyruvic acid	22.89			91	75	-0.35	0.48
M/50 α -ketoglutaric acid	22.65			91	85	-0.13	0.55
0.2% glucose + M/50 pyruvic acid	13.62			91	48.5	-1.56	0.92
0.2% glucose + M/50 α -ketoglutaric acid	16.29			91	26	-1.94	1.35

appearance being observed in the presence of glucose and the smallest in the presence of α -ketoglutaric acid. But when glucose and pyruvate or glucose and α -ketoglutaric acid are added together, amounts of NH_3 disappear which exceed the sum of the disappearances in, presence of either substrate alone. The highest rate of NH_3 disappearance and at the same time the highest values of amide-N are observed when glucose + α -ketoglutaric acid are added (Table XIV).

To explain this, it is necessary to anticipate some results which will be fully dealt with in further publications of this series. It will be shown that α -ketoglutaric acid is an intermediate in the oxidation of pyruvic acid. We assume, following Toennessen and Brinkmann [1930], a condensation of 2 mols. of pyruvic acid to form $\alpha\alpha'$ -diketoadipic acid. The next step however is probably not, as these authors assumed, hydrolysis to succinic acid and 2 mols. of formic acid but decarboxylation and oxidation, the product being α -ketoglutaric acid.



From the fact that the " NH_3 -binding mechanism" described above is regularly accompanied by an increase of amide-N it is concluded that the same reactions which lead to the disappearance of NH_3 and formation of glutamine in the presence of *l*(+)-glutamic acid can be arrived at from α -ketoglutaric acid, pyruvic acid or glucose. Krebs [1935, 2] has already shown that the synthesis of glutamine from *l*(+)-glutamic acid and NH_3 in brain and retina is very small in absence of glucose. The synthesis of glutamine from α -ketoglutaric acid and NH_3 equally requires the presence of glucose. Therefore the NH_3 -binding mechanism is smaller with α -ketoglutaric acid than with glucose, although the former is an intermediate in the process. The true effect of α -ketoglutaric acid is seen in the presence of glucose only. An analogy is the increasing effect of glucose and other substrates on urea formation in liver [Krebs and Henseleit, 1932].

Bicarbonate saline is a more favourable medium for the NH_3 -binding mechanism than phosphate saline.

VIII. The rôle of *l*(+)-glutamic acid in metabolism.

It is very improbable that the amount of glutamic acid supplied to the brain is sufficient to be of importance as fuel comparable with carbohydrate. The peculiar position of glutamic acid among the amino-acids suggests that it fulfils a specific function in connection with carbohydrate metabolism. The view is held that *in vivo* the glutamic acid deaminase of brain is concerned rather with synthesis than with breakdown of glutamic acid. This synthesis is part of an NH_3 -binding mechanism which leads from glucose *via* pyruvic acid and α -ketoglutaric acid to glutamic acid and glutamine. Glutamine is not the end product of the process, since it disappears also without liberating NH_3 [Krebs, 1935, 2]. Further work on these lines is in progress.

IX. SUMMARY.

The only amino-acid oxidised by brain is *l*(+)-glutamic acid which is oxidised to α -ketoglutaric acid and NH_3 and further to H_2O and CO_2 . The enzyme responsible for the oxidation of *l*(+)-glutamic acid to α -ketoglutaric acid and NH_3 does not attack *d*(-)-glutamic acid so long as it is bound in the cell or to some constituent of the cell, probably a lipid. In solution however the specificity is changed and *d*(-)-glutamic acid alone is oxidised.

The NH_3 derived from the deamination of $l(+)$ -glutamic acid disappears in secondary reactions leading to and beyond glutamine.

The reaction $l(+)$ -glutamic acid \rightarrow α -ketoglutaric acid is reversible. The existence of an NH_3 -binding mechanism is shown leading from glucose *via* pyruvic acid and α -ketoglutaric acid to $l(+)$ -glutamic acid, glutamine and further to an end product. The view is expressed that *in vivo* the glutamic acid deaminase is rather concerned with synthesis than with breakdown of glutamic acid.

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XCVI. THE EFFECT OF CERTAIN INGESTED FATTY OILS UPON THE COMPOSITION OF COW MILK FAT.

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THE observations of Drummond *et al.* [1923: 1924] and of Golding *et al.* [1926] that cod liver oil in the diet of lactating cows leads to reduction of the fat content of the milk have been confirmed by several other workers. McCay and Maynard [1935], who also state that ingestion of this oil causes muscular lesions in the animal, found that the effects of shark liver oil or salmon oil were so much less and so inconsistent that it is questionable whether they act like cod liver oil. These workers agreed with Golding [1928] that the specific effect of cod liver oil was not shown when the unsaponifiable fraction of that oil was given in the diet, thus pointing to the injurious or active component occurring as part of the triglycerides. Madsen *et al.* [1935] observed similar harmful results of cod liver oil in the diet of guinea-pigs, rabbits, goats and sheep.

In an attempt to ascertain which of the cod liver oil components were responsible for the diminished fat content of the milk, experiments were conducted at the National Institute for Research in Dairying in the early spring of 1934, in which the effect of cod liver oil was compared with that of rape or linseed oil in the diet. Rape oil contains nearly half its weight of erucic glycerides, *i.e.*, glycerides of an acid with a chain of 22 carbon atoms in the molecule, but monoethenoid as contrasted with the tetra- and penta-ethenoid unsaturation of the cod liver oil C_{20} and C_{22} acids. Linseed oil has a higher mean unsaturation than most cod liver oils, but the unsaturation is due solely to glycerides containing di- and tri-ethenoid acids of the C_{18} series (linoleic and linolenic), each of which forms about 40 % of the total fatty acids in linseed oil. Captain Golding, who informs us that ingestion of neither rape nor linseed oil caused any deficiency in the fat content of the milk, kindly placed at our disposal sufficient of the milk fats from these investigations, with corresponding control samples, to permit full analyses of the component acids by the ester-fractionation method. The present communication records the results of these analyses and of determinations of the proportion of fully-saturated glycerides in each of the fats.

The cows from which these milk fats were obtained received, in addition to the basal winter ration fed to the control animals, 4 oz. of either rape, linseed or cod liver oil twice daily for a week or more before taking the milk samples. (A fuller account of the experimental diets, in which the added oil was given twice daily, first in 2, then in 3 and finally in 4 oz. rations, was given in a recent communication by Dann *et al.* [1935].) The details of the milks, from which were prepared the six specimens of fat which we have studied, are given in Table I.

The general analytical characteristics of the corresponding milk fats are given in Table II.

Table I. *Sources of the six milk fats analysed.*

Reference no.	Cow or cows	Diet	Inclusive dates of collection of milk
1	Gertie 3	Control	p.m. March 14–a.m. March 16, 1934
2	Welcome 7	Control	Feb. 16–Feb. 19, 1934
3	Flora 16 and Flora 20	Control + linseed oil	p.m. March 27–p.m. March 28, 1934
4	Flora 16 and Flora 20	Control + rape oil	p.m. March 13–a.m. March 14, 1934
5	Portia 3	Control + cod liver oil	a.m. March 17–p.m. March 18, 1934
6	Lena 3 and Welcome 7	Control + cod liver oil	March 30–April 3, 1933

Table II. *General analytical characteristics.*

Milk fats from cows no. ...	1	2	3	4	5	6
Oil fed to cow ...	None	None	Linseed	Rape	Cod liver	Cod liver
Reichert-Meissl value	33.6	30.0	28.9	28.5	16.0	15.3
Polenske value	3.3	2.1	2.1	1.8	1.4	1.3
Kirschner value	24.3	23.8	22.4	21.7	13.9	13.7
Saponification equivalent	239.7	244.2	249.0	251.2	264.2	266.0
Iodine value	34.5	34.9	46.0	44.5	51.7	54.1

These figures show quite clearly that intake of fatty oil by the cows has caused considerable modification of the milk fat, but of course they indicate only the broad effects so produced and give little information as to the actual differences in the component acids, except that cod liver oil in the diet has approximately halved the amount of butyric and *n*-hexanoic acids in the milk fat and at the same time caused an increase in the total unsaturation of about 50 %, whilst the average equivalent of the mixed fatty acids has increased by about 25 units. Ingestion of linseed and rape oils apparently had little influence on the content of lower acids, but increased the mean unsaturation and equivalent to values similar to those characteristic of summer pasture butters [cf. Hilditch and Sleightholme, 1930; Dean and Hilditch, 1933, 1]. For more definite information as to the relative amounts of specific component acids, recourse to the more elaborate and tedious analytical methods by lead salt separation, ester fractionation *etc.* is essential, and it seemed well worth while to undertake this procedure on these six milk fats in view of the interesting features revealed by the data in Table II.

The methods employed have been described in detail previously [Hilditch and Jones, 1929; Hilditch and Sleightholme, 1930] and may be summed up as follows:

(a) The mixed fatty acids from about 400 g. (when available) of milk fat were first distilled in steam for 4–5 hours. The aqueous distillates were thoroughly extracted with ether and the extract dried over neutral anhydrous sodium sulphate. After removal of the ether, the residual volatile acids were fractionated direct. The acidity of the ether-extracted aqueous liquors, and of the ether *etc.* recovered by distillation up to 160°, was calculated as butyric acid, the compositions of the remaining fractions being calculated from their mean equivalents (with allowance for the small amount of unsaturation present in the final residue).

(b) The acids non-volatile in steam were submitted to the lead salt separation from alcohol and thereby divided into mainly saturated ("solid") and mainly unsaturated ("liquid") acids.

(c) Each group of acids, "solid" and "liquid", was converted into the corresponding neutral methyl esters, which were fractionally distilled in a high vacuum from a Willstätter bulb,¹ the first fraction (25–30 % of the whole) being refractionated whilst subsequent ones were collected in fractions of about 7–12 g. each. In the refractionation of the first primary fractions the distillates were divided into much smaller fractions (2–5 g.).

¹ We desire to emphasise the importance of employing some kind of simple fractionation device in these mixed ester distillations. The use of a Claisen bulb [cf. Ault and Brown, 1934] a, in our opinion, insufficient for the present purpose.

Table III. *Preliminary separation of the mixed fatty acids into (a) steam-volatile and (b) "solid" and "liquid" non-steam-volatile fatty acids.*

Milk fat no.	...	1	2	3	4	5	6
Oil fed to cow	...	Control	Control	Linseed	Rape	Cod liver	Cod liver
Weight of fat used (g.)		450.9	250.5	450.8	341	208	506
Total fatty acids (g.)		425.3	235.1	429.5	326.3	198.2	481.9
Steam-volatile acids (g.)		32.5	15.1	29.5	18.7	6.3	15.9
Lead salt separation:							
"Solid" acids (g.)		189.2	115.8	177.6	136.8	86.8	220.4
Corresponding methyl esters:							
Equivalent		275.8	275.4	276.0	281.8	276.2	278.6
I.V.		5.2	4.9	11.0	11.1	15.5	22.2
"Liquid" acids (g.)		203.6	106.2	222.4	170.8	105.1	245.6
Corresponding methyl esters:							
Equivalent		268.5	269.3	273.6	276.1	280.8	289.6
I.V.		68.1	71.7	78.6	76.7	81.0	84.4

Table IV. *Analyses of acids volatile in steam.*

Milk fat no.	...	1	2	3
Oil fed to cow	...	Control	Control	Linseed
Acidity (as butyric acid) in:		g. Equiv.	g. Equiv.	g. Equiv.
Ether-extracted aqueous solution		1.16 —	0.74 —	2.31 —
Recovered ether		0.55 —	0.26 —	0.66 —
Distillate fractions:				
1 (up to 100°)		0.29 —	0.11 —	0.27 —
2 (100-160°)		9.48 —	3.52 —	0.70 —
3		3.70 94.1	1.99 91.3	1.61 90.1
4		4.02 96.7	2.49 91.8	1.99 91.0
5		3.95 100.7	2.04 93.9	3.87 92.0
6		4.95 114.5	1.27 103.3	3.75 93.0
7		— —	1.30 116.2	6.92 96.3
8		— —	— —	2.68 112.5
9		— —	— —	2.15 121.8
10		— —	— —	1.26 139.4
Residue {		4.40 153.8	1.42 155.5	1.29 198.2
I.V.		— 9.9	— 12.2	— 22.3
Milk fat no.	...	4	5	6
Oil fed to cow	...	Rape	Cod liver	Cod liver
Acidity (as butyric acid) in:		g. Equiv.	g. Equiv.	g. Equiv.
Ether-extracted aqueous solution		0.35 —	0.21 —	0.84 —
Recovered ether		0.30 —	0.16 —	0.38 —
Distillate fractions:				
1 (up to 100°)		0.37 —	0.49 —	0.14 —
2 (100-160°)		1.68 —	1.90 —	1.68 —
3		1.07 91.8	0.87 95.0	1.59 91.4
4		1.08 92.1	0.74 97.3	2.19 91.6
5		2.17 92.2	0.70 108.1	1.65 91.8
6		1.99 92.5	— —	1.46 93.0
7		2.47 93.7	— —	1.27 95.5
8		2.18 95.8	— —	1.17 99.9
9		1.50 101.7	— —	2.13 115.7
10		1.25 114.2	— —	— —
11		0.86 121.5	— —	— —
Residue {		1.40 151.2	1.27 152.9	1.40 177.8
I.V.		— 10.6	— 11.5	— 20.3

(d) In certain cases, the mean equivalent of the saturated esters present in individual fractions was determined, after removal of all unsaturated esters by permanganate-acetone oxidation. This was carried out when it was desired to ascertain the mean unsaturation and equivalent of the unsaturated components also present in the fractions in question.

These detailed analyses show very clearly, especially with the milk fats from cows fed with rape or cod liver oil, the factors which have contributed to the marked changes in general analytical characteristics. In order to discuss the results adequately it is necessary to refer to specific parts of the ester fractionations and therefore also to quote the fractionation data somewhat more fully

Table V. *Methyl esters of "solid" acids.*

Milk fat no. ...	1			2			3		
Oil fed to cow...	Control			Control			Lanseed		
Fraction	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.
Primary fractionations.									
S 1	48.75	258.3	1.9	35.60	262.3	1.9	31.40	258.2	3.1
S 2	11.19	270.5	3.0	7.64	271.6	2.9	9.03	268.9	4.9
S 3	13.64	272.9	3.4	9.53	274.0	3.3	9.64	271.6	6.0
S 4	13.71	276.8	4.2	10.38	276.1	3.9	12.12	275.3	7.1
S 5	12.45	279.5	5.4	10.75	278.2	5.2	13.73	277.3	8.7
S 6	13.09	283.2	6.7	9.73	283.9	7.5	15.62	280.7	12.1
S 7	8.85	287.0	8.2	9.93	291.2	11.4	14.80	287.9	18.2
S 8	10.98	291.7	10.8	1.11	296.8	13.0	10.60	295.6	23.1
S 9	8.63	296.1	11.5	—	—	—	8.49	298.4	23.9
S 10	7.19	297.9	12.1	—	—	—	—	—	—
Residue	4.36	311.6	14.1	7.58	305.1	14.5	3.78	307.2	25.6
Refractionations of primary fractions S 1.									
S 11	2.80	237.1	0.6	4.42	248.0	0.9	2.62	241.8	0.6
S 12	2.87	243.1	0.6	4.02	250.5	0.9	2.90	246.4	0.5
S 13	4.80	247.7	0.7	5.38	255.3	0.8	3.74	250.6	0.6
S 14	6.45	251.0	0.7	4.91	261.4	0.9	5.10	256.7	1.3
S 15	8.40	256.7	0.9	4.82	269.7	1.4	5.42	264.6	2.5
S 16	7.80	265.9	1.5	5.57	273.5	2.4	3.72	269.6	4.8
S 17	5.85	273.0	2.9	—	—	—	—	—	—
Residue	4.98	283.1	6.6	2.92	281.5	7.9	3.99	279.4	11.8
Milk fat no. ...	4			5			6		
Oil fed to cow...	Rape			Cod liver			Cod liver		
Fraction	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.
Primary fractionations.									
S 1	43.44	262.8	3.5	31.42	255.8	4.3	45.37	265.6	6.2
S 2	12.71	276.4	6.0	7.72	275.6	7.9	9.54	272.4	11.1
S 3	12.07	279.7	7.7	7.20	276.6	10.2	12.37	277.7	13.1
S 4	12.19	282.8	9.9	7.82	280.3	13.0	13.90	278.9	16.0
S 5	12.91	287.7	14.0	6.31	284.6	19.9	11.68	280.6	20.4
S 6	11.98	295.6	18.4	6.92	292.1	27.4	13.83	284.5	27.0
S 7	8.86	300.8	21.4	—	—	—	11.98	289.4	37.0
S 8	6.00	306.6	23.7	—	—	—	14.98	298.4	50.1
Residue	6.87	330.8	37.2	10.56	304.6	39.6	10.39	315.2	62.8
Refractionations of primary fractions S 1.									
S 11	4.49	243.9	1.1	2.94	250.0	1.4	4.01	247.7	1.2
S 12	4.80	252.2	2.0	2.80	252.2	1.3	4.26	253.3	1.4
S 13	5.92	256.1	1.2	4.81	258.9	1.6	5.10	256.0	2.0
S 14	7.00	263.0	1.9	3.82	267.4	2.6	7.59	263.1	2.8
S 15	7.03	270.0	2.9	4.26	271.5	4.0	5.56	268.1	4.0
S 16	5.45	276.2	5.1	3.06	275.0	7.0	4.55	271.5	6.5
S 17	—	—	—	—	—	—	5.13	273.3	9.7
Residue	4.95	284.8	11.2	2.69	274.7	17.9	4.94	282.4	25.5

than has been the recent custom in communications from this laboratory, where it has been considered sufficient to give only the final percentage compositions obtained for the component acids of a fat. The details of the analyses are therefore summarised in Tables III, IV, V and VI. The weights of the fats used and the corresponding total weights of fatty acids accounted for, were as shown in Table III.

Table VI. *Methyl esters of "liquid" acids.*

Milk fat no. ...	1			2			3		
Oil fed to cow ...	Control			Control			Linseed		
Fraction	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.
Primary fractionations.									
L 1	53.38	216.7	22.6	31.23	227.6	33.5	41.51	232.2	37.4
L 2	12.81	275.5	68.8	6.37	280.3	71.8	11.87	283.5	82.4
L 3	16.56	289.3	87.8	8.99	286.1	80.1	12.14	289.8	88.4
L 4	17.30	292.4	94.7	10.46	291.7	87.1	16.21	291.3	94.0
L 5	10.14	293.2	98.2	11.14	293.4	91.9	22.08	293.0	97.8
L 6	22.62	294.8	101.2	8.85	296.0	94.0	11.96	294.8	98.7
L 7	12.01	299.7	106.5	10.00	297.6	96.3	9.73	295.3	98.9
L 8	—	—	—	6.19	298.7	96.9	8.76	297.2	101.0
Residue	7.13	341.3	112.6	6.46	325.5	99.7	10.00	323.8	106.1
	—	303.9*	109.2*	—	301.8*	100.7*	—	304.8*	103.9*
Refractionations of primary fractions L 1.									
L 11	2.64	163.7	3.2	2.78	169.4	6.7	3.08	170.0	5.8
L 12	2.54	174.0	6.2	3.09	187.5	13.4	2.55	188.8	12.0
L 13	5.48	185.4	9.1	4.10	209.8	11.5	2.35	196.4	11.7
L 14	5.20	193.1	9.1	5.14	232.4	18.1	3.22	212.7	10.6
L 15	6.09	213.3	11.2	3.76	248.9	27.6	4.45	228.0	14.0
L 16	10.53	227.9	11.9	4.11	263.4	48.0	4.90	244.5	23.7
L 17	6.49	244.4	19.5	—	—	—	4.90	254.5	34.8
L 18	6.81	263.3	49.8	—	—	—	3.50	268.0	56.0
L 19	—	—	—	—	—	—	4.03	282.5	80.0
Residue	4.20	295.7	86.2	5.15	290.1	86.8	4.62	292.1	94.2
Milk fat no. ...	4			5			6		
Oil fed to cow ...	Rape			Cod liver			Cod liver		
Fraction	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.	g.	Sap. eq.	I.V.
Primary fractionations.									
L 1	48.25	240.5	40.8	35.21	252.0	56.8	45.44	251.7	53.5
L 2	23.78	289.7	87.9	16.08	291.4	90.4	12.00	290.0	89.0
L 3	12.91	293.7	92.1	8.78	294.5	95.3	12.58	290.2	93.7
L 4	15.44	295.5	94.1	9.32	294.2	95.5	13.38	296.4	96.9
L 5	16.35	296.6	95.7	10.09	298.3	98.1	10.99	296.8	99.6
L 6	11.49	297.2	96.5	6.11	302.3	98.4	15.91	298.5	102.3
L 7	11.53	299.7	98.3	5.79	308.1	101.6	7.56	299.7	103.2
L 8	—	—	—	—	—	—	7.46	303.3	105.5
L 9	—	—	—	—	—	—	7.42	306.4	106.9
L 10	—	—	—	—	—	—	6.02	317.3	113.5
Residue	13.35	330.6	99.9	8.16	320.7	99.0	8.16	315.8	118.5
	—	320.1*	—	—	310.0*	—	—	318.0*	110.1*
Refractionations of primary fractions L 1.									
L 11	3.40	172.4	5.8	2.50	186.2	11.7	3.04	182.7	10.5
L 12	3.20	190.6	13.8	4.62	222.3	21.1	4.37	215.5	17.2
L 13	5.05	215.1	12.1	10.17	250.6	42.1	5.45	238.0	33.3
L 14	13.81	244.2	21.9	10.31	283.6	80.7	5.89	252.5	37.1
L 15	15.70	279.5	66.5	—	—	—	7.85	265.9	55.9
L 16	—	—	—	—	—	—	5.36	280.4	77.1
L 17	—	—	—	—	—	—	4.97	288.9	89.7
Residue	3.43	293.4	89.8	4.35	298.2	94.7	4.57	296.9	99.3

* Residual esters, freed from unsaponifiable.

Without entering into the full details of the calculations used in evaluating the complicated series of data in Tables III-VI, the general principles which were employed may be briefly enumerated:

(i) *Esters of unsaturated C_{18} acids.* The unavoidable assumption has been made that in each case the unsaturated esters of the C_{18} acids distil throughout in the same proportions (e.g., in milk fat 1, these esters have been taken throughout as of i.v. 101.2; in milk fat 4, of i.v. 94.1 etc.). This assumption, although strictly not correct (di-ethenoid esters distilling slightly more readily than mono-ethenoid esters in the lower-boiling ranges), is not very far from the truth. In previous papers these acids have finally been computed as mixtures of oleic and "linoleic" acids. The occurrence of ordinary linoleic acid in milk fats has recently been discussed at some length [Bosworth and Brown, 1933; Eckstein, 1933; Green and Hilditch, 1935]. The result of these investigations leaves us of the opinion that practically the whole of the unsaturated C_{18} acids are either mono- or di-ethenoid. Consequently, for the purpose of these analyses, we feel justified in making no alteration in the presentation of the results, except that it is more appropriate to term the diethenoid acid or acids "octadecadienoic" and to avoid the use of the specific connotation "linoleic".

(ii) *C_{20} acids present in normal cow milk fats.* The final fractions of the "solid" esters have almost always possessed equivalents slightly higher than correspond with esters of C_{18} acids and fractional percentages "as arachidic acid" have consequently been recorded. Whether this is in reality arachidic acid, $C_{20}H_{40}O_2$, or traces of still higher saturated acids, we cannot say; it might be a mono-ethenoid acid or acids, but the more unsaturated arachidonic acid should not appear in the "solid" acids. The latter acid or acids, in the small amounts (0.2-0.3%) recorded by Brown and Sutton [1931], has, in our previous butter fat analyses, been left in the residue from the primary fractionation of the liquid esters [cf. Hilditch, 1934, p. 784] and it has not been necessary to include it in our calculations. Curiously (and somewhat unfortunately in view of the fact that some of the present fats are further complicated by the presence of ingested unsaturated C_{20} and C_{22} acids), all the milk fats (including the controls) now studied contain definitely more of these unsaturated C_{20} or C_{22} acids than has previously been observed: so much so, that the equivalents not only of the residual "liquid" esters but also of the fraction or fractions immediately preceding are definitely higher than that of C_{18} esters (ca. 295.5). (Cf., for example, Table VI, milk fat no. 2 (control), primary fractions L 7, L 8 and residue, equivalents respectively 297.6, 298.7 and 301.8.) A similar state of affairs has been observed in the case of some pig depot fats [Banks and Hilditch, 1932; Dean and Hilditch, 1933, 2], and the method of approximate calculation used for these pig fat analyses has also been employed in the present work. Apparently the unsaturated C_{20-22} acids in the present group of milk fats amount to nearly 1% of the component acids, apart from any of these acids derived from fatty oils added to the diet.

(iii) *Rape oil diet.* In this instance abnormally high equivalents characterised the final fractions (S 7, S 8, S 9, L 8) from both "solid" and "liquid" esters. Examination of the saturated esters present in S 7-9 (obtained after oxidation with permanganate in acetone, *v. infra*, Table VIII) led to the conclusion that methyl erucate was present in the unsaturated portion of these fractions, and allowance for this was therefore made in the calculations.

(iv) *Cod liver oil diet.* Nothing very abnormal was noticed in the fractions obtained by the distillation of the "solid" esters from milk fats 5 and 6, but the later fractions from the "liquid" esters (no. 5, L 5-8 and no. 6, L 5-11) were marked by equivalents which rose well over that of C_{18} esters and, at the same

time, by increases in I.V. as compared with that of the C_{18} esters. Clearly, unsaturated acids of the C_{20-22} series were present in much greater quantity than in the other fats and had passed into the milk fat from the ingested cod liver oil.

With the modifications referred to above, the composition of the various groups of acids (steam-volatile, "solid", "liquid") derived from each milk fat was calculated from the data in Tables III-VI by the usual methods, and the final results are given, in the form of weight and also of molar percentages, in Table VII.

Table VII. *Component acids of cow milk fats nos. 1-6.*

Milk fat no.	...	1	2	3	4	5	6
Oil fed to cow	...	Control	Control	Linseed	Rape	Cod liver	Cod liver
Acid		%	%	%	%	%	%
(i) Weight percentages.							
Butyric		4.4	4.4	4.2	3.6	2.0	2.1
<i>n</i> -Hexanoic		2.2	1.4	2.0	1.6	0.6	0.9
<i>n</i> -Octanoic		2.4	1.8	1.3	1.3	0.9	0.5
<i>n</i> -Decanoic		3.8	1.9	2.3	1.6	1.3	1.2
Lauric		4.4	3.1	3.1	2.8	4.1	3.1
Myristic		10.9	9.3	8.4	8.6	6.7	6.4
Palmitic		23.1	27.5	21.8	18.1	23.2	22.7
Stearic		12.6	12.2	9.9	13.8	8.2	6.7
As Arachidic		0.7	1.0	0.6	0.5	0.6	0.6
Oleic		28.9	33.1	39.3	39.7	42.8	43.3
As Octadecadienoic		5.6	3.1	5.9	3.7	4.5	4.8
As C_{20-22} unsaturated		1.0	1.2	1.2	1.0	5.1	7.7
As Erucic		—	—	—	3.7	—	—
(ii) Molar percentages.							
Butyric		11.5	11.7	11.2	9.9	5.7	6.1
<i>n</i> -Hexanoic		4.3	2.8	4.1	3.4	1.2	2.0
<i>n</i> -Octanoic		3.7	2.9	2.1	2.3	1.6	0.8
<i>n</i> -Decanoic		5.0	2.6	3.1	2.2	1.9	1.8
Lauric		5.0	3.6	3.6	3.4	5.1	3.9
Myristic		10.8	9.5	8.6	9.1	7.4	7.1
Palmitic		20.5	25.2	20.0	17.0	22.8	22.4
Stearic		10.1	10.0	8.2	11.7	7.3	6.0
As Arachidic		0.5	0.8	0.4	0.4	0.5	0.5
Oleic		23.3	27.4	32.8	34.0	38.3	38.8
As Octadecadienoic		4.5	2.6	5.0	3.2	4.1	4.4
As C_{20-22} unsaturated		0.8	0.9	0.9	0.8	4.1	6.2
As Erucic		—	—	—	2.6	—	—

The general results of the investigation are readily revealed by inspection of Table VII: ingestion of cod liver oil has resulted in each case in reduction of the proportion of butyric-lauric acids in the milk fats to about half of the amount present in normal milk fats, whilst the milk fat component acids include 5-8 % (wt.) of unsaturated C_{20-22} acids, as compared with about 1 % in cases in which the cows received no cod liver oil. On the other hand, ingestion of neither linseed nor rape oil led to any comparable effect (although certain minor effects, almost within the range of experimental error, might possibly be attributed to the rape oil); consequently neither high mean unsaturation of the ingested (linseed) oil, nor the presence therein (rape) of high proportions of the mono-ethenoid C_{22} (erucic) acid, produces the results specific to cod liver oil.

Before discussing the data in further detail, it is desirable at this point to record the results of some experiments which were made in order to ascertain whether palmitoleic acid (which also occurs in quantity in cod liver oil) had passed, like the fish oil C_{20-22} acids, into the milk fat to any extent. If so, this

acid would be present in "liquid" ester fractions immediately preceding the approximately individual C_{18} ester fractions. Some of these fractions were therefore submitted to permanganate-acetone oxidation in order to isolate the saturated esters present and determine the equivalent of the latter. At the same time, similar oxidations were carried out on certain lower fractions the mean equivalent of which indicated a high concentration of esters of C_{14} acids.

The results of these experiments (and also of oxidations of certain "solid" ester fractions undertaken to ascertain whether erucic or gadoleic acids were present in specific cases) are given in Table VIII.

Table VIII. *Saturated esters present in certain ester fractions (Tables V-VI).*

Milk fat no.	Origin to cow	Ester fractions				Saturated
		No.	g.	Sap. eq.	I.V.	Sap. eq.
(i) "Liquid" ester fractions.						
4	Rape	L 14	13.81	244.2	21.9	249.0
4	"	L 15	15.70	279.5	66.5	263.4
4	"	L 2	23.78	289.7	87.9	270.4
5	Cod liver	L 13	10.17	250.6	42.1	249.6
5	"	L 14	10.31	283.6	80.7	267.9
5	"	L 2	16.08	291.4	90.4	265.3
6	"	L 15, L 16, L 17	18.18	276.1	71.4	261.8
		L 2, L 3	24.58	290.1	91.4	290.2
(ii) "Solid" ester fractions.						
4	Rape	S 7, S 8	14.86	303.1	22.3	301.2
4	"	S 9	6.87	330.8	37.2	327.2
5	Cod liver	S 7	10.56	304.6	39.6	303.2
6	"	S 8	14.98	298.4	50.1	297.4
6	"	S 9	10.39	315.2	62.8	320.6

The data in Table VIII for fractions of equivalent 280-290 suggest that, in all three milk fats, the equivalent of the unsaturated esters is somewhat lower than 295.5 (methyl esters of C_{18} acids)—in other words, that small amounts of palmitoleic ester are also present. (It should be pointed out that the proportion of saturated ester in fractions such as L 2, with i.v. 85-90, is very small and its isolation and the accurate determination of its equivalent a matter of some difficulty.) In fractions 4, L 14, and 5, L 13, with mean equivalents of 244.2 and 250.6, the proportion of saturated ester was fairly large, and the interesting feature emerges that the mean equivalent of the unsaturated esters here is also low (240-250), indicating the presence of detectable amounts of methyl tetradecenoate. Evidently therefore the small proportions of decenoic and tetradecenoic acids observed in milk fats by Grün *et al.* [1922; 1924] and by Bosworth and Brown [1933] have appeared, as would be expected, in the lower-boiling fractions of the refractionated first primary fraction of the "liquid" esters. We know however that methyl oleate also passes in small quantities into ester fractions of the average equivalents in question, and it is therefore quite beyond the capacity of the fractionation procedure to attempt any precise estimate of the amounts of these minor unsaturated components. Yet, if we assume for the moment that fractions L 11 and L 12 contain only methyl decenoate as unsaturated ester, and that succeeding fractions contain only either tetradecenoate, or the latter with hexadecenoate, or hexadecenoate with C_{18} unsaturated esters, the results so calculated (while probably exaggerating the

amounts of these lower unsaturated acids) will afford at all events a comparison of the hexadecenoic (palmitoleic) contents of milk fats when cod liver oil has or has not been included in the diet. These figures also show the relatively small effect which allowance for these minor components has upon the estimated amounts of the major component acids. Table IX gives the result of such alternative computations for milk fats 4 (rape oil) and 5 (cod liver oil). (Unfortunately the L 11 *etc.* fractions in the two control fats 1 and 2 were not collected in quantities suitable for oxidation experiments, but, since palmitoleic acid is not present in rape oil, the use of milk fat 4 for the present purpose presents no objection.) In Table IX the weight percentages of the component acids are calculated (a) without allowance for lower unsaturated acids (as in Table VII) and (b) allowing as described above for decenoic, tetra- and hexadecenoic acids in the specified ester fractions of the L 11 *etc.* groups.

Table IX. *Alternative calculations of component acids in milk fats 4 and 5 ("o wt.).*

Milk fat no.	...	4		5	
Oil fed to cow	...	Rape		Cod liver	
Acid		(a)	(b)	(a)	(b)
		o/o	o/o	o/o	o/o
Butyric		3.6	3.6	2.0	2.0
n-Hexanoic		1.6	1.6	0.6	0.6
n-Octanoic		1.3	1.0	0.9	0.6
n-Decanoic		1.6	1.5	1.3	1.3
Lauric		2.8	1.8	4.1	0.9
Myristic		8.6	8.1	6.7	8.4
Palmitic		18.1	20.3	23.2	25.4
Stearic		13.8	13.8	8.2	8.2
As Arachidic		0.5	0.5	0.6	0.6
As Decenoic		—	0.2	—	0.5
As Tetradeceenoic		—	1.3	—	1.4
As Hexadecenoic		—	2.4	—	3.3
As Oleic		39.7	36.0	42.8	37.8
As Octadecadienoic		3.7	3.3	4.5	3.9
As C ₂₀₋₂₂ unsaturated		1.0	1.0	5.1	5.1
As Erucic		3.7	3.6	—	—

These figures have several points of interest. They show that, with the extreme assumption that unsaturation in the lower ester fractions is due entirely to unsaturated acids of lower molecular weight than oleic, the only notable difference is a drop of about 4% in the estimated content of oleic acid and a rise of about 2% in that of palmitic acid; the rest of the acids are affected either not at all, or to no significant degree. The actual amounts of decenoic and tetradeceenoic acids suggested by the calculations accord well with the minimum amounts which Bosworth and Brown [1933] concluded must be present (0.18% decenoic and 0.87% tetradeceenoic). If the computation is accepted for these acids, it would be illogical not to apply it to hexadecenoic (palmitoleic) acid, but Bosworth and Brown were unable to confirm Grün's earlier statement that this acid also occurred in butter fat.

We do not consider that the ester-fractionation procedure, as we have used it, can be rightly pushed to this extreme of arithmetical deduction, which in the ordinary way we should not adopt; the case might be otherwise if much larger amounts of fatty acids were employed and it was consequently feasible to perform a number of refractionations of the lower-boiling "liquid" esters. Our

primary purpose in this discussion has been to discover whether palmitoleic acid (present to the extent of about 15% in cod liver oil) has, like the highly unsaturated C_{20} and C_{22} acids of that oil, passed into the milk fats of cows receiving the oil and, whatever may be the limits of accuracy of the alternative calculation adopted, it is clear that the palmitoleic acid content of milk fat 5 is not markedly greater than that of milk fat 4, whereas the unsaturated C_{20-22} acids of fat 5 show a fivefold increase over those of fat 4.

GENERAL DISCUSSION.

A general comparison of the fatty acid compositions in Table VII may now be made.

"Control" milk fats 1 and 2. These are of the usual type for winter-fed cattle, with comparatively low oleic and octadecadienoic contents and the usual proportions of butyric *etc.* acids. The palmitic acid content is rather different in the two fats, that of fat 1 being the more normal, although the higher figure of fat 2 is not extreme; the palmitic and oleic acid contents of normal butters may vary inversely over a range of 5-6%, perhaps depending upon the age of the cow [Dean and Hilditch, 1933, 1].

"Linseed oil" milk fat 3. The only marked effect of the ingestion of linseed oil is an increase in the content of oleic acid. Octadecadienoic (or other polyethenoid C_{18}) acid is present in only slightly, if at all, increased proportion compared with the control fats or other normal milk fats. Although the unsaturated C_{18} acids yielded on oxidation very small amounts (1-2%) of tetrahydroxystearic acid, M.P. 155°, they failed to give either the petroleum-insoluble tetrabromostearic acid or the ether-insoluble hexabromostearic acid characteristic of the linoleic and linolenic acids which are present in large quantity in linseed oil. Thus there is no evidence of passage of the highly unsaturated linseed oil acids into the milk, the lower saturated acids of the milk fat are unaffected and the only notable effect is the increase mentioned in oleic acid. The stearic acid content is a little lower than in the control fats.

"Rape oil" milk fat 4. Here again the chief effect is an increase (quantitatively similar to that with linseed oil) in the proportion of oleic acid. The lower saturated acids are perhaps slightly below the normal amount, as is also palmitic acid, whilst the stearic acid is slightly increased. The reduced proportions mentioned may however be merely the additive result of infiltration of the rape oil acids which, as shown by the small proportion of erucic acid present, have passed into the milk to a small extent. Nevertheless, the large proportion (nearly 50%) of erucic (C_{22}) acid combined in the rape oil has not materially affected the composition of the milk fat, which is very similar to that in the linseed oil feeding experiment.

"Cod liver oil" milk fats 5 and 6. The chief points to be observed in these fats are: (i) the enormous reduction in the lower saturated acids, (ii) a proportionately similar increase in oleic acid, (iii) no great difference from the normal in the amount of octadecadienoic (or other polyethenoid C_{18}) acid, (iv) the presence of about 5% of C_{20-22} unsaturated acids instead of the 1% of these acids found in fats 1-4 and (v) the apparent absence of any palmitoleic (hexadecenoic) acid, although this is almost as prominent a component of the cod liver oil acids as the C_{20-22} acids. These features are seen clearly in Table X, in which the differences between the molar percentages of the component acids are given for the respective mean values of the "control" milk fats 1 and 2 and the "cod liver oil" milk fats 5 and 6.

Table X. *Differences in component acids of milk fats from cows on "control" and "cod liver oil" diets.*

(Mean values of fats 1 and 2, 5 and 6 respectively.)

Acid	"Control" 1 and 2 % (mol.)	"Cod liver oil" 5 and 6 % (mol.)	Difference	Percentage alteration
Butyric	11.6	5.9	5.7	49
<i>n</i> -Hexanoic	3.6	1.6	2.0	55
<i>n</i> -Octanoic	3.3	1.2	2.1	64
<i>n</i> -Decanoic	3.8	1.9	1.9	50
Lauric	4.3	4.5	0.2	-
Myristic	10.2	7.2	3.0	29
Palmitic	22.8	22.6	0.2	-
Stearic	10.0	6.7	3.3	33
As Arachidic	0.6	0.5	0.1	—
Oleic	25.4	38.5	13.1	+ 52
As Octadecadienoic	3.6	4.2	0.6	—
C ₂₀₋₂₂ unsaturated	0.8	5.2	4.4	+ 550

It should be noted that, even if the increase in C₂₀₋₂₂ acids were interpreted as the result of infiltration of cod liver oil glycerides as a whole (the absence of increase in palmitoleic acid of course shows this is not the case), this would correspond with the admixture of at most 20 % of cod liver oil in the milk fat; and would by no means account for the disproportionate diminution in the saturated acids (other than palmitic). The analytical data disclose definitely an influence on the milk fat metabolism quite beyond the limits of any additive or dilution effect.

The palmitic acid figures in Table VII are also interesting. Cod liver oil has not affected the normal content of this acid in the milk fat, linseed oil has perhaps caused a slight fall, and rape oil has produced a definite fall to below any figure which we have previously observed in a butter fat. Rape oil contains only 2 % of palmitic acid, linseed oil about 6 % and cod liver oil 15-18 %; and the above data might be interpreted in favour of the view that a part, at least, of animal palmitic acid (which forms about 25 % of all depot and milk fats yet examined) is derived by assimilation of preformed vegetable palmitic acid.

Fully-saturated glyceride content of milk fats 1-6. Since the content of fully-saturated glycerides in milk fats and in cow, sheep and pig depot fats has a quite different relation to the total proportion of saturated to unsaturated acids in the whole fats from that which obtains in seed fats or fish oils, and since this relation, when plotted graphically, gives a series of points which, for the group of fats mentioned, lie about a smooth curve, it was felt desirable to determine the content and mean equivalent of the fully-saturated glycerides in each fat of the present series, in order to ascertain whether the cod liver oil diet had also produced an anomalous effect on the glyceride structure of the milk fats. The experimental data obtained are summarised in Table XI and have also been plotted (Fig. 1) together with all the data obtained previously for cow milk fats, depot fats and pig depot fats.

Insufficient material was available for detailed analyses of the component acids in the fully-saturated glycerides, but their saponification equivalents point to a similar composition in each of the fats 1-4; Hilditch and Sleightholme [1931] observed close similarity in the compositions of the fully-saturated glycerides in milk fats (of varying total unsaturation) from cows fed in summer or winter conditions. The "cod liver oil" milk fats 5 and 6 however evidently contain fully-saturated glycerides which have less of the lower saturated acids and more

Table XI. *Fully-saturated glyceride contents of milk fats 1-6.*

Milk fat no.	Oil fed to cow	Whole fat			Fully-saturated glycerides		
		I.v.	Sap. eq.	Saturated acids % (mol.)	% (wt.)	% (mol.)	Sap. eq.
1	Control	34.5	239.7	71.4	37.1	40.4	221.0
2	"	34.9	244.2	69.1	31.1	34.2	224.5
3	Linseed	46.0	249.0	61.3	22.2	24.8	222.4
4	Rape	44.5	251.2	59.4	22.4	25.3	225.0
5	Cod liver	51.7	264.2	53.5	15.3	17.2	235.7
6	"	54.1	266.0	50.6	12.8	14.6	235.1

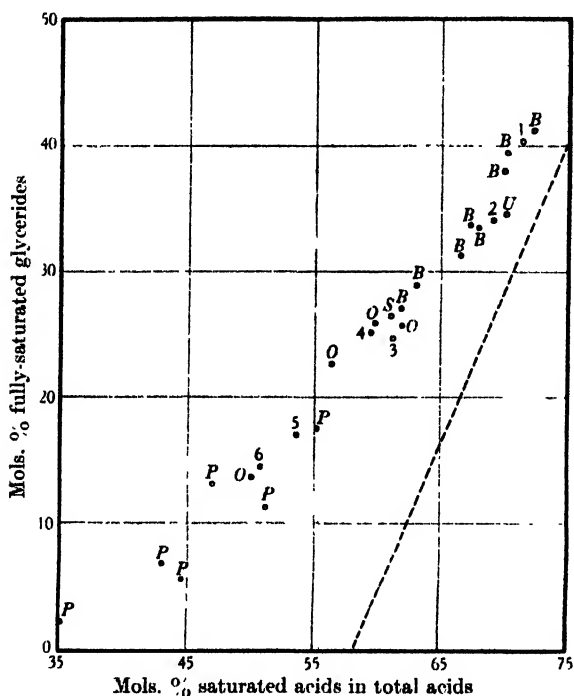


Fig. 1. Data from previous work: *B*, cow milk fats; *U*, Buffalo milk fat; *S*, sheep depot fat; *O*, ox depot fat; *P*, pig depot fat. Data from present work: 1, 2, cow milk fat (control); 3, cow milk fat (linseed oil); 4, cow milk fat (rape oil); 5, 6, cow milk fat (cod liver oil).

palmitic (and probably myristic), as is not unnatural in view of the deficiency of the former acids in the fats as a whole.

At the same time, as will be seen from Fig. 1, the proportion of fully-saturated glycerides in these "cod liver oil" milk fats has exactly the same relation to the total saturated acids in the fat as in the remainder of the butter fat and the corresponding depot fat series. The whole group lies about a regular curve which would intersect the horizontal axis at a point corresponding with about 30 % of total saturated acids (*i.e.* the approximate content of palmitic (with myristic) acid characteristic of all animal depot fats). It forms a series quite distinct from most vegetable and some other animal fats, in which the glycerides are assembled on "evenly distributed" lines, having negligible contents of fully-saturated glycerides unless their total saturated acids amount to more than about 60 %

of the whole, and thereafter giving a relation expressed by the broken line in Fig. 1. These special relationships in the milk and depot fats of the animals concerned are explicable [cf. Hilditch and Sleightholme, 1931; Banks and Hilditch, 1931; 1932; Hilditch and Stainsby, 1935] on the hypothesis that the unusual amounts of fully-saturated components are the result of formation of stearic glycerides or glycerides of lower saturated acids from oleic glycerides (which would have existed primarily mainly in the form of "evenly distributed" mixed palmito-oleo-glycerides). In spite of the marked differences in the relative amounts of their component acids, the milk fats from cows receiving cod liver oil in the diet still conform to this typical relationship with regard to their fully-saturated components. Indeed their positions on the curve in Fig. 1 emphasise the fact that, from this aspect of glyceride structure, there is complete uniformity in type between cow milk fats and ox, sheep or pig depot fats.

The present work has enabled us to define more closely the factor in cod liver oil which is responsible for the marked alteration in the milk fat. Previous investigators [Golding, 1928; McCay and Maynard, 1935] have agreed that the triglycerides of the oil contained the injurious or active component; our analyses show that, of the specific cod liver oil acids not present in the other oils used in the feeding trials, palmitoleic acid has not passed appreciably into the milk fat, whereas the highly-unsaturated C_{20-22} acids appear therein in some quantity. There is thus evidence of specific retention by the mammary tissues of certain fatty acids and not others: whilst these highly-unsaturated C_{20-22} acids are comparatively readily absorbed, linolenic and (very largely) linoleic acids of linseed oil cannot be detected, although erucic acid, $C_{22}H_{42}O_2$, of rape oil is probably accepted to a minor extent. Lovern [1932; 1934] has found evidence for similar specificity in fatty acid absorption in the adipose tissues of fish and marine mammalia. Retention of C_{20-22} glycerides of cod liver oil by the mammary gland does not of course imply in itself that this is the causative factor of the abnormal milk fat composition; nevertheless, we consider that, if the above general hypothesis of the elaboration of the characteristic milk fat structure may be provisionally accepted, there is a simple and logical reason why this should in fact be the case.

The production of stearic from preformed oleic glycerides (in depot fats and to some extent in milk fats) involves simple reduction or hydrogenation; that of the lower saturated glycerides of the milk fats is a much more complex process, the mechanism of which cannot yet be surmised but obviously would involve oxidation as well as reduction. It is well known that the highly unsaturated C_{20} and C_{22} acids of the "clupanodonic" group (or their esters) are avid acceptors of both hydrogen and oxygen; for example, they absorb atmospheric oxygen, or undergo catalytic hydrogenation, far more rapidly than monoethenoid fatty acids or esters. Their presence in a biological oxidation-reduction system, such as must be conceived necessary for the production of glycerides of butyric-decanoic and stearic acids, where otherwise glycerides of oleic acid would appear, would therefore be expected to retard this part of the metabolic process. It is unnecessary to postulate that in such circumstances alternative products of metabolism should be observed. Selective adsorption alone by the enzyme system concerned of the highly-unsaturated glycerides would disturb and hinder its normal action. In other words, the function of the highly-unsaturated glycerides would be that of a "catalyst poison", entirely analogous, for example, to the prevention of atmospheric oxidation of an unsaturated fat by an "anti-oxidant", or to the retardation of the union of ethylene and hydrogen at a nickel or other catalytic surface in presence of carbon monoxide, when the

retarding agent may be chemically unaltered, or much more slowly altered, by concurrent catalytic action upon itself.

This conception of the effect of the presence of highly-unsaturated glycerides in the mammary gland accounts for the nature of the component acids observed in milk fats 5 and 6, and also for the fact that the general relationship of fully-saturated glycerides to total saturated acids therein still remains unaltered as compared with other milk and depot fats. Table X shows that, after allowing for the influx of C_{20-22} acids, all that has happened is a substantial increase above normal in the proportion of oleic acid while the formation of butyric-decanoic acids has been greatly retarded and also, to a smaller extent, that of stearic and myristic acids. The explanation offered is also in harmony with the results of the other feeding experiments. With linseed oil, which also possesses high mean unsaturation, no evidence was found of absorption of tri- and di-ethenoid acids into the milk fat, and it therefore seems that the mammary tissue is not permeable to these particular glycerides. The metabolic processes are consequently undisturbed except that the abnormal intake of oleic acid (also present in the linseed oil) is reflected in a higher percentage of that acid at the expense of slight reductions in stearic and one or two of the other saturated acids. A similar state of affairs is seen in the rape oil experiment, although erucic glycerides have passed to some extent into the milk fat; but erucic is a monoethenoid acid and would have no more intense effect than oleic as regards selective adsorption by the metabolic enzymes.

Similarly, shark liver oil which, according to McCay and Maynard [1935], is innocuous, is an Elasmobranch oil: the glycerides of these contain unsaturated acids of the C_{16} , C_{18} , C_{20} , C_{22} and C_{24} series which however are almost wholly monoethenoid in contrast to the C_{20} and C_{22} acids of Teleostean fish [Hilditch and Houlbrooke, 1928]: so that, on the present hypothesis, the results observed are those which would be expected. Salmon oil, on the other hand, contains highly-unsaturated C_{20-22} acids, but McCay and Maynard found no abnormal effect in its ingestion, this observation being the only one not in harmony with our suggested explanation. McCay and Maynard's studies were focussed on changes in the muscular tissues, another instance in which oxidation-reduction processes are involved, and the influence of highly-unsaturated C_{20-22} glycerides here may perhaps be similar in character to that suggested in the mammary gland.

Finally, the rapidity with which the milk fat (and its content in the milk) reverts to normal when the addition of cod liver oil to the diet is abandoned [Golding, 1928; Dann *et al.*, 1935] is wholly in accord with the view that the enzyme system concerned in producing the characteristic butter-glycerides has been temporarily and partially put out of action owing to selective adsorption of the highly-unsaturated C_{20-22} glycerides.

SUMMARY.

Detailed studies were made of the component acids in milk fats from cows receiving (a) a normal winter ration and (b) the same ration with additions of cod liver, linseed or rape oils. Cod liver oil had a marked effect, the lower saturated acids of the milk fats being reduced to half the normal content, while the proportion of oleic acid was much increased, and a certain amount (5-7 %) of highly-unsaturated C_{20-22} acids was present. Polyethenoid unsaturation in the C_{18} acids was not more than normal and palmitoleic acid had not been appreciably absorbed from the cod liver oil. The effect of linseed oil was merely to increase the proportion of oleic acid; linolenic acid was not detected in the milk

fat and linoleic acid only in very small amounts, the mean unsaturation of the C_{18} acids and the amount of lower saturated acids were normal. Rape oil had a similar effect to linseed oil, but small amounts of erucic glycerides were present in the milk fats. The relationship between fully-saturated glyceride content and proportion of total saturated acids in the milk fats was normal (*i.e.* of the type characteristic of cow milk and depot fats) in all cases.

The investigation shows that some of the highly-unsaturated C_{20-22} glycerides of cod liver oil (but not its palmitoleic acid or the linolenic and linoleic acids of linseed oil) pass into the milk fats; and it is suggested that selective adsorption of these highly-unsaturated compounds by the enzymes responsible for the elaboration of typical cow milk fats retards their normal function and causes the observed effects. This hypothesis is a corollary to that previously suggested to explain the abnormal production of fully-saturated glycerides in milk and certain animal depot fats as compared with vegetable fats and some other animal depot fats, and is also in harmony with the results obtained on adding linseed, rape or shark liver oils to the diet of lactating cows.

We desire to thank Captain Golding and the Director of the National Institute for Research in Dairying for placing the milk fats and details of the diet *etc.* of the cows at our disposal, and Messrs Lever Brothers, Limited, whose Research Studentship in this Department was held by one of us (H. M. T.) during part of the investigation.

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XCVII. HEXOSEPHOSPHATES PRODUCED BY HIGHER PLANTS.¹

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THE fact that phosphates stimulate the respiratory sugar metabolism in higher plants suggests that the formation of phosphoric esters may occur as part of this process. Stoklasa *et al.* [1904–1913] obtained cell-free zymase-like preparations from peas, beet-roots, potatoes *etc.*, which actively fermented sugars. Bodnár [1916] confirmed these results under strictly aseptic conditions, since some investigators had attributed Stoklasa's findings to bacterial contamination. Evidence of phosphorylation was first given by Bodnár [1925] who demonstrated that inorganic phosphate disappeared when added to ground peas in presence of toluene. His results were confirmed by Zalesky and Pissarjewski [1927] who did not however regard this process as a necessary step in the respiration and attributed the stimulation to alkalinity of the phosphate rather than to the formation of phosphoric esters. More recently, Rao [1935] has prepared active cell-free aqueous extracts by plasmolysing fermenting peas with light petroleum and states that in such extracts the decomposition of sugar and the phosphorylation are concomitant processes.

While Bodnár's findings indicated the possible parallelism between carbohydrate metabolism in the higher plants and in yeast or muscle [Harden, 1932; Bodnár and Tankó, 1929], little evidence was available regarding the nature of the compounds formed during the phosphorylation. It is known that when preparations of germinating peas, beans, barley *etc.* are allowed to act upon the hexosediphosphoric acid of Harden and Young products are formed which are characteristic of sugar breakdown in yeast or muscle [Neuberg and Gottschalk, 1924; 1925; Neuberg and Kobel, 1929; 1930; 1934, 1; Baba, 1935]. Moreover, with phosphoglyceric acid as a substrate, pyruvic acid is formed [Neuberg and Kobel, 1934, 2].

Barrenscheen and Albers [1928] found that the acid-soluble phosphorus increased during assimilation in irradiated *Elodea canadensis* and during germination of rye. A phosphoric ester was isolated by Barrenscheen and Pany [1930] from the phosphorylated products formed when *Elodea* was kept in a dilute solution of sugar and inorganic phosphate for an irradiation period of 4 hours. In its composition (Ba, P) the ester resembled a hexosemonophosphate, but it differed from the Robison ester [Robison, 1922] in possessing a much lower reducing power (iodimetric, 3.2 %; H.J. 3.5 %). These investigators also isolated the barium salt of a phosphorylated octaamylose from the germ plants of wheat. This substance gave a high dextrorotation, $[\alpha]_D^{20} + 44.9^\circ$, and after hydrolysis by takadiastase of its magnesium salt, yielded a hexosemonophosphoric ester having H.J. 22.6 % and $[\alpha]_D^{20} - 4.3^\circ$. On hydrolysis by bone phosphatase the monophosphate yielded fructose, which was identified by means of its methylphenylosazone. The authors also found that the synthetic phosphorylated

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starch, prepared by the method of Kerb, yielded on partial hydrolysis by taka-diastase a compound probably identical with the phospho-octaamylose obtained from plants. These experiments, carried out under conditions approaching the physiological, strongly support the view that hexosephosphates are formed by higher plants. The crude monophosphate isolated from *Elodea* by Barrenscheen and his collaborators resembles none of the esters characteristic of yeast and muscle, and these investigators considered that the phospho-octaamylose of wheat occurred preformed in the starch molecule. Whilst plants may have their own individual peculiarities in combining phosphate with sugar, the results of Neuberg and his co-workers, referred to above, seem to suggest that the phosphoric esters known to take a leading part in the sugar-breakdown by yeast and muscle may also form a part of the phosphorylated compounds produced by higher plants.

The aim of the present research has been to approach the problem of phosphorylation in higher plants from the preparative side and to isolate and identify as far as possible the compounds produced by pea preparations under the conditions devised by Bodnár.

EXPERIMENTAL.

Ground peas were prepared according to Bodnár [1925]. Removal of the proteins was effected by trichloroacetic acid and the phosphorus was estimated by Briggs's method, as modified by Martland and Robison [1926]. Marrow peas (No. 110, supplied by G. Kontsek, Ltd.) yielded active (phosphorylating) preparations simply by grinding the seeds, the skins being removed by sifting through grenadine (metal sieves were avoided). Preparations of Victoria and sugar peas did not phosphorylate unless the peas were first allowed to swell in water; the skins being removed and the seeds dried at 30–32° before being ground and sifted.

The behaviour of the marrow pea-flour indicated that bacterial contamination during the swelling process was not responsible for the phosphorylation. Moreover, if the swelling was allowed to proceed in the presence of chloroform or toluene instead of water alone, equally active preparations were obtained. The pea-flour retained its activity for several months. Using the marrow pea-flour, the addition of inorganic phosphate equivalent to 10 mg. P per g. of flour was found to provide the most suitable experimental conditions; increasing the phosphate to 15 mg. P per g. did not appreciably increase the amount of esterification, whilst the larger proportion of unesterified phosphate interfered with the subsequent isolation of phosphoric esters.

The optimum p_H for esterification.

0.3 g. of the pea preparation was added to 1.5 ml. portions of the buffer solution (veronal-acetate buffer [Michaelis, 1931]), each containing 0.9 mg. P as inorganic phosphate. The tubes (10 ml.), to which a drop of toluene was added, were placed in a desiccator containing water and toluene and kept at 32°; several tubes were prepared at each p_H . The inorganic phosphate was estimated at 0, 2 and 5 hours after the addition of 3 ml. of H_2O and 2.5 ml. of 10 % trichloroacetic acid. The p_H was determined on a separate series of tubes of similar composition, using the quinhydrone electrode. As is shown in Table I, the optimum p_H for the disappearance of inorganic phosphate lies slightly on the acid side of neutrality. The observed optimum may be an apparent one, for 1 mg. of organic phosphorus was present in the pea preparation used, and some of this may have been hydrolysed at the more alkaline p_H values.

Table I. *Effect of p_H on the disappearance of inorganic phosphate from pea preparations.*

Veronal-acetate buffer. The inorganic P values are calculated for 0.3 g. pea used in each experiment. Estimation of p_H was carried out by means of the quinhydrone electrode at 22°.

Time	p_H					
2 min.	6.16	6.63	6.96	7.63	8.02	8.34
2 hours	6.14	6.54	6.77	7.43	7.87	8.24
5 hours	6.12	6.49	6.69	7.30	7.78	7.99
mg. inorganic P/0.3 g. preparation						
0 hours	1.16	1.17	1.17	1.17	1.16	1.17
2 hours	1.06	1.00	0.99	1.10	1.19	1.21
5 hours	0.98	0.91	0.86	1.07	1.23	1.28

Rate of phosphorylation.

20 g. of pea-flour in a 200 ml. flask were stirred to a paste with 40 ml. H_2O or phosphate solution (204 mg. P; p_H 7.2) and 2 ml. of toluene. The pea-flour was stirred at intervals and the experiment was stopped at the time indicated by the addition of 70 ml. of H_2O and 70 ml. of 10% trichloroacetic acid. The protein precipitates were washed with 50 ml. 3% trichloroacetic acid.

The filtrates were adjusted with NaOH to p_H 8.4 and the precipitates were filtered off, washed and dried (Fraction 1).

Basic lead acetate solution was added in slight excess to the filtrates. The washed basic lead precipitates were suspended in water and decomposed with H_2SO_4 ; the $PbSO_4$ was centrifuged down and washed repeatedly with acidified H_2O . The centrifugates and washings were adjusted to p_H 8.4 with baryta (without the removal of the $BaSO_4$) and the phosphoric esters precipitated by the addition of 2.5 volumes of 96% alcohol. The dried precipitate (A), after the total and inorganic P had been estimated, was repeatedly extracted with 15 times its weight of H_2O , added in portions. The solution was again precipitated with alcohol; the dried precipitate was extracted with H_2O as before, and 33% alcohol, sufficient to give a 10% solution, was added. The insoluble portion was centrifuged down; the P remaining in the centrifugate represented Fraction 2, *i.e.* the neutral barium salt soluble in 10% alcohol.

Fraction 3, the barium salt insoluble in 10% alcohol, is represented by the P content of precipitate A, less that of Fraction 2. The reducing sugars in the filtrate from the basic lead precipitation were determined by the Hagedorn-Jensen method and were calculated as glucose.

The results, summarised in Table II, show that a considerable accumulation of esterified phosphate occurs when the pea-flour preparations are incubated for periods of from 35 to 70 hours in the presence of added inorganic phosphate. The amount of reducing sugar fell in each experiment where phosphorylation took place, suggesting that phosphoric esters of the sugars were formed. The phosphorylation is greatly in excess of that taking place in the autofermentations. The phosphoric esters produced are found mainly in Fraction 3 and to a much less extent in Fraction 2. Fraction 1, which remained at a constant level during the autofermentations, decreased slightly in the experiments in which inorganic phosphate was added; this fraction had a phosphorus content of 16–18% and gave a negative Molisch reaction. The lead salt was insoluble in acetic acid and the fraction gave the Fischler and Kürten [1932] reaction for phytin.

Table II. *Distribution of organic P in fractions obtained from phosphorylation experiments with pea-flour.*

20 g. of pea preparation were used in each experiment and all figures are calculated for the total trichloroacetic filtrate.

Fractions: 1, insoluble sodium salt.

2, soluble barium salt.

3, sparingly soluble barium salt.

In Exps. 3, 5, 7 the figures in brackets show the increase in esterified P resulting from the addition of inorganic phosphate.

In the autofermentations the p_H dropped from 6.8 to 6.4; in those with added inorganic phosphate from 7.0 to 6.6.

The reducing power was estimated in the filtrates from the basic lead precipitates.

Exp.	mg. inorg. P added	C	Dura- tion hours	mg. P in								Reduction H.J. mg. as glu- cose
				Trichloroacetic filtrate		Frac- tion 1	Filtrate of fraction 1		Frac- tion 2	Fraction 3		
				Org.	Inorg.		Org.	Inorg.		Org.	Inorg.	
1	-	--	0	65	17	48	18	16	4	5	12	190
2	-	32	8	79	8	51	30	7	11	8	7	373
3	204	32	8	130	160	40	90	160	14 (3)	50 (42)	155	172
4	-	32	35	81	11	47	33	12	14	11	10	416
5	204	32	35	273	18	40	229	22	32 (18)	159 (148)	26	205
6	-	21	70	86	4	48	38	4	16	14	3	374
7	204	21	70	274	13	42	232	13	28 (12)	163 (149)	17	148

The effects of fluoride and monochloroacetic acid.

The formation of hexosephosphates was strongly inhibited by 0.002M NaF, and a similar result was obtained with monochloroacetic acid. The hydrolysis in N HCl, at 100° of the trichloroacetic filtrate from an experiment with 0.0002M NaF (which did not inhibit phosphorylation), did not reveal any evidence of the accumulation of more difficultly hydrolysable esters in comparison with the controls.

Isolation and identification of the phosphoric esters formed.

As Table II shows, Fraction 3, the sparingly soluble barium salt, constituted by far the greater part of the phosphoric esters formed during the experimental period. For isolation and identification of the esters, the phosphorylated products obtained from several experiments (carried out under the same conditions as Exp. 7) were combined and fractionated according to the general procedure already outlined above. The basic lead salt was decomposed with H_2SO_4 at 0° to avoid possible hydrolysis, and the neutral barium salt was prepared.

Fraction 3 was further purified by formation of the soluble acid barium salt by the addition of HCl to p_H 3, the insoluble $BaSO_4$ being separated by centrifuging. It was precipitated from the centrifugate at p_H 4 by the addition of 4 volumes of 96% alcohol. The filtrate from this precipitation was adjusted to p_H 8.4, and the precipitate obtained was combined with Fraction 2.

Fraction 2 was further purified by four reprecipitations from its aqueous solution by alcohol. The portion insoluble in 10% alcohol was purified by precipitation of the acid salt with alcohol and then combined with Fraction 3.

From 100 g. of pea-flour, to which 1 g. of P as inorganic phosphate had been added, about 6 g. of the crude acid barium salt of Fraction 3 and 1 g. of the crude neutral barium salt of Fraction 2 were obtained.

The diphosphate fraction (Fraction 3). This fraction was still contaminated with inorganic phosphate, which was removed by means of magnesia mixture, and the acid barium salt again precipitated (yield, 5 g.). There was no separation of phosphoglycerates when a small amount of the ester was kept at 0° in solution in 20 % alcohol. The ester was further purified by two reprecipitations of the acid barium salt (yield, 3.5 g.). Part of it was converted into the neutral barium salt by dissolving in water, adjusting to p_H 8.4 with baryta and precipitating with an equal volume of alcohol. The analysis of the substance is shown in Table III, No. 1. (All figures are calculated on the dry weight; 90 minutes' drying at 110°

Table III. *Analyses of neutral Ba salts of the phosphoric esters isolated from the phosphorylation experiments.**

No.	Source of Ba salt	Ba %	P %	Reducing power as glucose, %		Fructose (Seli- vanoff) %	$[\alpha]_D^{20}$ + 3.0° ($c = 28.6^{10}_0$)
				H.J.†	Iodine		
1	Fraction 3, purified	-	10.0	12	1.4	8	
1 a	Monophosphate obtained from No. 1 by acid hydrolysis	-	7.8	34	2.0	25	+ 2.3
2	Fraction 2, crude	33.8	5.9	21	24.0		+ 4.6
3	Fraction 2, purified through brucine salt	34.6	7.7	29.5	28	8	+ 7.3
3 a	Mother-liquor of brucine salt	--	2.7	10	14	2	---
4	No. 3 after removal of aldoses by bromine oxidation	-	7.8	22	0.5	17	+ 4.3
5	Fraction 2, originally present (0 hour exp.)	--	1.9	9	22	2	- 17.2
6	Fraction 2, from autofermentation (21°, 70 hours)	--	2.9	9	20	1	- 6.9
* Analyses of hexosephosphates for comparison:							$[\alpha]_D^{20}$ + 3.0°
	Hexosediphosphate ¹		10.0	12	1.5	9	+ 2.0
	Fructose-6-phosphate ¹	-	7.73	35	1.6	22	+ 2.3
	Robison ester ²		7.85	30	25.0	6	+ 14.4
	Glucose-6-phosphate ³	-	7.86	36	45.7	(0.5)	+ 20.6
	Mannose-6-phosphate ³	-	7.87	36	28.5	(0.3)	+ 3.6
	Fructose-1-phosphate ⁴	-	7.85	26	0.9	24	- 39.0
	Galactose-6-phosphate ⁵	---	7.86	28	41.0	(0.7)	+ 30.0

† Determined with addition of 0.5 ml. of 0.5 *N* NaOH.

¹ Macleod and Robison [1933].

² Robison [1922].

³ Jephcott and Robison [1934].

⁴ Tankó and Robison [1935].

⁵ Grant [1935].

in the Pregl micro-dryer.) The analysis of the neutral barium salt agrees closely with that of fructosediphosphate. The rotation was measured in acid solution and calculated for the neutral salt.

Phenylosazone. The phenylhydrazine salt of the phenylosazone was prepared and reprecipitated from alcohol-chloroform; the phospho-osazone obtained, dried over H_2SO_4 *in vacuo* had m.p. 152.5–153°. (Found: P, 5.52 %; $C_{24}H_{31}O_7N_6P$ requires P, 5.68 %.)

Rate of hydrolysis. The rate of hydrolysis of the acid salt in *N* HCl at 100° is shown in Table IV, where it is compared with that found by Macleod and Robison [1933] for 1:6-diphosphofructofuranose; the figures are in excellent agreement.

The monophosphate prepared by the fractional hydrolysis of the acid salt (6 min. in *N* HCl at 100°) was analysed and found to consist of the Neuberg

Table IV. *Hydrolysis in N HCl at 100° of hexosediphosphate isolated from pea products.*

Time (min.)	Hydrolysis %		$k \cdot 10^3$	
	A	B	A	B
	Diphosphate from pea products (0.455 mg. P per ml.)	1:6-Diphospho- fructofuranose* (0.454 mg. P per ml.)		
5	23.7	23.3	22.3	23.0
10	36.5	36.8	15.9	16.8
25	53.5	54.4	9.0	9.4
60	69.5	68.8	5.2	4.7
120	84.1	83.5	4.7	4.6
180	91.4	90.2	4.4	3.7
300	96.8	96.6	3.6	3.8

* Macleod and Robison [1933].

ester, fructose-6-phosphate (Table III, 1a). The results show that the hexosediphosphate isolated from Fraction 3 and formed during the phosphorylation taking place with the pea preparations used is identical with the Harden-Young ester formed by yeast preparations, *i.e.* 1:6-diphosphofructofuranose.

The hexosemonophosphate fraction (Fraction 2). The analysis of the crude Fraction 2 suggested that it consisted partly of hexosemonophosphates. The reducing power was in good agreement with the P, but the ratio Ba/P was 1.3 and the substance contained about 2% N, only partly precipitated by phosphotungstic acid. By fractional precipitation with alcohol a fraction was obtained between 40–70% alcohol, which had the following analysis: P, 3.9%; Ba/P 1.8; H.J. 12%; I₂, 38%. Thus the crude fraction contained a compound of low P and relatively high Ba content (suggesting a carboxylic group) and possessing a high iodine value.

For further purification, Fraction 2 was converted into the brucine salt [Robison and King, 1931] and 90% of the original phosphorus was found in fractions having P, 2.9–2.5% (brucine hexosemonophosphate requires 2.95%). The fractions of lower P content, after recrystallisation from 90% alcohol, gave P 2.9%. The fractions were combined and recrystallised from water. During the separation of the first crop crystals were formed resembling those of brucine fructose-1-phosphate [Tankó and Robison, 1935]. The combined crops (P, 2.85–2.94%) were reconverted into the barium salt and reprecipitated three times in the usual manner. From 1 g. of the crude barium salt of Fraction 2 0.4 g. of the purified monophosphate was obtained. The residue left in the mother-liquors was converted into the barium salt and precipitated several times from its aqueous solution with 96% alcohol. The analyses (Table III, Nos. 3, 3a) show that a hexosemonophosphate was obtained in fairly pure state and separated from the material in the mother-liquors, which had a low P and a high iodine value. The monophosphate obtained (N-free; Ba/P = 1) differed from the Robison ester chiefly in its lower dextrorotation, in spite of its somewhat higher iodine value.

Removal of aldosemonophosphates by oxidation with bromine. A portion of the pure monophosphate (0.3 g.) was oxidised with bromine to remove the aldose components as described by Tankó and Robison [1935]. The analysis of the product (0.05 g.), after the separation of the phosphohexonates, is given in Table III, No. 4. The rate of hydrolysis of this non-aldose portion in N HCl at

Table V. *Hydrolyses of hexosemonophosphates obtained from pea products.*

Time min.	Before bromine oxidation (0.297 mg. P per ml.)			After bromine oxidation (0.294 mg. P per ml.)	
	Hydrolysis %		$k \times 10^3$	Hydrolysis %	$k \times 10^3$
	Found	Calculated*			
5	—	—	—	24.0	23.8
35	20.2	20.0	2.8	49.6	5.7
60	—	—	—	58.0	3.1
180	34.8	33.8	0.60	73.6	3.4
630	48.4	49.5	0.23	82.8	0.41
2160	75.5	—	0.21	—	—
5160	93.7	—	0.19	—	—

* Assuming the composition to be: 54% of glucose-6-phosphate, $k = 0.23 \times 10^3$; 8% of mannose-6-phosphate, $k = 0.29 \times 10^{-3}$; 38% of the compound obtained after bromine oxidation whose hydrolysability is shown in the last two columns.

100° is compared with that for the original monophosphate in Table V. The observed properties of this non-aldose component are difficult to explain on the assumption that it consists only of the two known fructosemonophosphates (fructose-6- and fructose-1-phosphate). The observed laevorotation is too small for the high initial rate of hydrolysis, assuming this to be due to fructose-1-phosphate: the low Selivanoff value suggests the presence of a non-fructose derivative, as does the rapid fall in the rate of hydrolysis after 3 hours. Fructose-6-phosphate ($k = 4.2 \times 10^{-3}$ [Robison, 1932]) under the above conditions would be hydrolysed to the extent of 28.7% in 35 min., 82.5% in 180 min.: the non-aldose component of the present monophosphate is hydrolysed 49.6% in 35 min., 73.6% in 180 min. The excellent solubility of the non-aldose portion, the correct phosphorus and low iodine values rule out the possibility that unchanged aldose-monophosphate or phosphohexonates can be responsible for these discrepancies. Fructose-6-phosphate is a most likely constituent of the product, accompanied by a more readily hydrolysable compound (possibly fructose-1-phosphate) and one less readily hydrolysable. More attention will have to be directed to the compounds originally present in the pea-flour used, or formed during the auto-fermentation process (see Table III, Nos. 5, 6, for analyses), which possess low P and Selivanoff values, high iodine values, are laevorotatory and are hydrolysed by acids at a rate comparable with that of glucose-6-phosphate.

Phosphohexokinase in pea preparations.

It has been shown that the Robison ester of yeast fermentation consists of a mixture of the 6-phosphates of glucose, fructose and mannose [Robison, 1932] and that a phosphohexokinase occurs in yeast and in muscle, bone and other tissues capable of causing this equilibrium mixture to be formed from any one

Table VI. *Demonstration of phosphohexokinase in pea preparation.*

mg. pea prepara- tion per mg. ester P	Substrate: fructose-6-phosphate.				
	Time (min.)	...	2	5	15
44.6	Aldose %		33	58	62
22.3	Aldose %		21	38	60

The original I_2 value of the fructose-6-phosphate was 2.5%.

Concentration of ester (Na-salt) 0.02 molar: $p_H = 7.2$.

The figures for aldose are based on the I_2 values, less the amount due to the pea preparation, and are given as percentages of the total calculated hexose in the substrate.

of the hexosemonophosphates [Lohmann, 1933; Tankó and Robison, 1935; Patwardhan and Robison, unpublished results]. The proportion of 62 % aldose and 38 % non-aldose esters in the monophosphate at present under investigation suggested that such an enzyme might also be present in the plant preparation used.

50 or 100 mg. of the pea-flour were added to 1.4 ml. portions of a solution of the sodium salt of fructose-6-phosphate (1.6 mg. ester-P per ml.). The mixtures were kept at room temperature and the action was stopped by the addition of trichloroacetic acid. The iodimetric reducing power was estimated on the neutralised trichloroacetic filtrate. The iodine value of the pea-flour used was low in comparison with the changes taking place and did not alter significantly during the period of the experiment. The ketose ester (which had only 2.5 % iodine value) was converted to the extent of 62 % into aldose ester. The change was complete in 15 min. and no hydrolysis of the ester took place in that time; nor was fructose-diphosphate hydrolysed when added in place of the monophosphate. There was no appreciable increase in iodine value when fructose and inorganic phosphate were used.

DISCUSSION.

These results indicate that under the optimum conditions (p_H , temperature and concentration of inorganic phosphate) for the rate of phosphorylation of dried pea preparations, a hexosediphosphate and hexosemonophosphates accumulate. These esters have been isolated and their properties compared with those of the phosphoric esters known to play a part in the carbohydrate metabolism of yeast and muscle.

The diphosphate which constituted the major portion of the phosphorylated products was the Harden-Young ester, 1:6-diphosphofructofuranose. No evidence was obtained of the formation of phosphoglycerates. The monophosphate fraction was a mixture of aldose and ketose esters resembling the Robison ester, but containing other components, possibly fructose-1-phosphate and a difficultly hydrolysable non-aldose ester which is being further investigated. The phosphorylation in the pea preparation was inhibited by NaF and by monochloroacetic acid. The preparations contained a phosphohexokinase.

The evidence obtained supports the view that phosphorylation takes place in higher plants, with the production of the same phosphoric esters as are formed in yeast or muscle. In addition esters are present which differ in constitution from the known phosphoric esters and which may be related to the reserve materials of the pea preparations, since these were the source of the hexose constituents of the phosphoric esters produced. The pea preparations used in the experiments hydrolysed hexosediphosphate at very low rates. Preparations obtained in a similar way from fresh green peas hydrolysed the diphosphate very rapidly, and this may explain why such preparations did not produce any accumulation of phosphoric esters. Preparations from germinating marrow peas behaved similarly to those from fresh green peas. Further work will be carried out to define the nature of the monophosphates formed when the hexosediphosphate is hydrolysed by these preparations.

SUMMARY.

1. The optimum conditions of the experiments devised by Bodnár [1925] were investigated in order to increase the amount of organic phosphorus compounds formed from added inorganic phosphate by pea preparations in the presence of toluene, without any extra carbohydrate.

2. The method of separation of the phosphorylated products is described. These compounds are phosphorylated derivatives of the sugars.

3. The greatest part, over 90 % of the esterified phosphorus, has been identified as the Harden-Young ester, 1:6-diphosphofructofuranose.

4. The monophosphate complex has been separated from the other fractions and purified by means of the brucine salt. The pure monophosphate resembled the Robison ester but had a somewhat lower dextrorotation and there was some evidence that this was due to fructose-1-phosphate. Another difficultly hydrolysable non-aldose ester was present and is being further investigated.

5. The presence of phosphohexokinase in the pea preparations has been demonstrated; this enzyme converts fructose-6-phosphate into a mixture of 62 % aldose- and 38 % non-aldose-esters.

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XCVIII. DISTRIBUTION OF VITAMIN C IN DIFFERENT PARTS OF COMMON INDIAN FOODSTUFFS.

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(Received December 25th, 1935.)

SINCE vitamin C was identified with ascorbic acid several attempts have been made to assay the vitamin in foodstuffs by modifications of Tillman's dichlorophenolindophenol titration method. Bacharach *et al.* [1934] by examining oranges, tangerines and lemons found that vitamin C was present in gradually diminishing amounts from the skin inwards and they suggested that vitamin C was photosynthesised at the skin. A systematic investigation of a large number of foodstuffs was undertaken in this laboratory in order to ascertain the distribution of vitamin C in different parts of the foodstuff. Bessey and King [1933] and Birch *et al.* [1933] give methods of extracting the vitamin from foodstuffs for titration with the indophenol reagent. The author used the following modified method in order to obtain strictly comparable results.

5-10 g. of the substance, or as much as is available, are taken in a mortar and 10 ml. of 20% trichloroacetic acid are added. The mass is then ground into a fine paste with clean sand previously treated with acids, washed and dried. The pasty mass is then stirred up with water and filtered on a Büchner funnel with suction. The residue is transferred to the mortar, 2.5 ml. of trichloroacetic acid are added and the mass is ground up and filtered. The residue is washed 2 or 3 times with water and the combined filtrates made up to 50 ml.; 0.5 ml. of the dichlorophenolindophenol solution (*M*/100 approx.) is taken and 10 ml. of glacial acetic acid are added following upon the observation of Ghosh and Guha [1935] that the addition of the acetic acid inhibits the decolorising action of trichloroacetic acid. The red solution thus obtained is then titrated with the filtrate from a burette until the dye is decolorised. The titration is timed by a stop-watch and is finished in about 1½ min. The dye is next titrated with a solution of ascorbic acid, at about the same rate. The ascorbic acid is finally titrated with *N*/100 iodine solution.

Generally the ascorbic acid contents of the skin and the flesh of fruits and vegetables have been assayed. In some cases the ascorbic acid content of leaves and in two cases that of the seeds have also been determined. It will be seen that in all cases the ascorbic acid is more concentrated in the skin than in the flesh (Table I). The result thus confirms the finding of Bacharach *et al.* It was expected that the leaves of the root vegetables would give higher values than the skin or the flesh and this expectation was realised except in the case of the radish where the difference is within the experimental error.

Quite recently Ranganathan [1935] reported that he had confirmed the results of Bacharach *et al.* in some but not in all cases. McHenry and Graham [1935] also found that in cucumber the ascorbic acid was more concentrated in the skin than in the flesh, but in turnip the reverse was the case, and they advanced an argument to explain this apparent discrepancy. We have, however, failed to

Table I.

Serial No.	Name of food	Scientific name	Ascorbic acid content (mg./g.)			
			Skin	Flesh	Leaves	Seeds
1	Patal	<i>Momordica dioica</i>	0.444	0.25	0.289	0.09
2	Pumpkin	<i>Cucurbita maxima</i>	0.1	0.051	0.1	0.03
3	Ninua	<i>Luffa aegyptica</i>	0.102	0.023	-	---
4	Banana	<i>Musa</i>	0.062	0.052	-	---
5	Plantain	<i>Musa</i>	0.486	0.451	---	---
6	Pear	<i>Pyrus communis</i>	0.072	0.016	---	---
7	Apple	<i>Pyrus malus</i>	0.533	0.202	---	---
8	Radish	<i>Raphanus sativus</i>	0.41	0.16	0.43	---
9	Turnip	<i>Brassica campestris</i>	0.259	0.172	0.393	---
10	Gourd	<i>Lagenaria vulgaris</i>	0.671	0.141	-	-
11	Potato	<i>Solanum tuberosum</i>	0.56	0.363	-	-
12	Tamarind	<i>Tamarindus indica</i>	0.222	0.135	-	-
13	Guava	<i>Psidium guajava</i>	1.854	1.02	---	---
14	Plum	<i>Zizyphus jujuba</i>	3.2	0.337	-	-
15	D. (another var.)		4.0	0.470	-	-
16	Tomato	<i>Lycopersicon esculentum</i>	0.57	0.39	-	-
17	Carrot	<i>Daucus carota</i>	0.75	0.31	-	-
18	Green chilli	<i>Capsicum</i>	3.9	3.3	-	-
19	Sweet potato	<i>Ipomoea batatas</i>	0.987	0.911	0.993	---
20	Kohl rabi	<i>Brassica oleracea</i>	1.552	0.97	3.264	---
21	Brinjal	<i>Solanum melongena</i>	0.8	0.267	---	---

find this departure from normal in any case. On the other hand we have found that when the fruit or the vegetable is fresh and young the ascorbic acid in the skin is invariably more concentrated than in the flesh; but if the material is dried up, that is, if it is stored for sometime the ascorbic acid of the skin may be less concentrated than that of the flesh (Table II). The obvious explanation

Table II. *Ascorbic acid contents of skin and flesh of dried and stored foods (mg. per g.).*

	Name	Skin	Flesh
1	<i>Luffa aegyptica</i> (Ninua)	0.022	0.029
2	Plantain	0.092	0.099
3	Sweet potato	0.703	0.90

is that the ascorbic acid of the skin, being exposed to atmospheric action, begins to deteriorate while that in the flesh, being protected by the outer coating, remains unharmed and intact for considerably longer periods. The failure of Ranganathan to detect ascorbic acid in the skin of the plantain or banana may be due to his method of extraction with 8% acetic acid. Ghosh and Guha [1935] have adduced evidence that trichloroacetic acid fails to extract all the ascorbic acid from germinating mung. McHenry also points out that one extraction with trichloroacetic acid fails to extract all the ascorbic acid. We have also found that our method of extracting twice with trichloroacetic acid (20%) failed to extract all the ascorbic acid from some materials.

Examination of animal foods has revealed that the liver is the richest in ascorbic acid whilst muscle is the poorest (Table III).

Table III. *Ascorbic acid contents of animal foods (mg. per g.).*

	Name	Liver	Kidney	Heart	Muscle	Bone-marrow	Milk
1	Goat	0.263	0.176	0.077	0.066	0.088	0.085
2	Hilsa (<i>Clupea Hilsa</i>)	0.48	—	—	0.278	—	—

SUMMARY.

It has been found that vitamin C is more concentrated in the skin of fruits and vegetables than in the edible portion. In the case of root vegetables vitamin C is in the greatest concentration in the leaves. Animal foods gave the following result in regard to their vitamin C contents:

liver > kidney > bone-marrow > milk > heart > muscle.

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XCIX. CARCINOGENIC ACTION AND ABSORPTION AND FLUORESCENCE SPECTRA OF 1:2-BENZPYRENE.

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(Received December 4th, 1935.)

ISOLATION of the active principles of carcinogenic tars [Cook *et al.*, 1933; Maisin and Coolen, 1934; Barry *et al.*, 1935] was accomplished by using their characteristic fluorescence spectra [Hieger, 1930; 1933]. It seemed interesting to ascertain at what stage in the synthesis of 1:2-benzpyrene [Cook and Hewett, 1933], the most active carcinogenic constituent of tar, the characteristic fluorescence and the physiological activity might appear, and also whether the fate of 1:2-benzpyrene in the organism could be followed by this specific fluorescence (as attempted by u.v. absorption spectra with 1:2:5:6-dibenzanthracene [Chalmers, 1934]). To this end we record here the spectra of some precursors of 1:2-benzpyrene as well as of 1:2-benzpyrene itself.

Carcinogenic activity.

Our purified synthetic 1:2-benzpyrene (m.p. 176.5–177.5°; pierate, m.p. 197–198°) was more greenish yellow in colour than was Cook's product although of nearly identical m.p. The colour of the pure 1:2-benzpyrene obtained by Winterstein and Vetter [1934] by chromatographic fractionation was also yellow-green. Our product, tested on mice [Sannié *et al.*, 1936], showed carcinogenic activity entirely concordant with that observed by Barry *et al.* [1935].

Ultra-violet absorption spectra.

Absorption spectra were determined by using a large Féry spectrograph to obtain photographs which were microphotometered as already described [Sannié, 1934; 1935]. For 1:2-benzpyrene absorption was similar to that described by Mayneord and Roe [1935] in alcoholic solutions. In hexane solutions, bands were observed at 2544, 2840, 2966, 3290, 3460 and 3640 Å., with finally a wide band at 3800, resolvable into three lines at 3787, 3807 and 3829 Å. Less distinct inflections were observed about 2620 and 2725 Å. 4'-Keto-1':2':3':4'-tetrahydro-1:2-benzpyrene gave absorption maxima as follows: 2403, 2618, 2727, 3130, 3269, 3424, 3644, 3846 and 4079 Å. Comparison of this spectrum with the spectrum of 1:2-benzpyrene (and especially with the subsidiary inflections observed at 2620 and 2725 Å.) makes it probable that our synthetic 1:2-benzpyrene, in spite of purification to m.p. 176.5–177.5°, still contained appreciable quantities of its ketonic precursor.

Fluorescence spectra.

Fluorescence was excited in an apparatus similar to that of Bourguel [1933] for Raman spectra by using intense light from a mercury arc (Gallois lamp filtered through Wood's glass (Corning)). A Huet spectrograph with an average

dispersion of about 13 Å. per mm. at 4000 Å. was used, opened at F/8, with Fulgur Guillemot (sensitised to 5000 Å.) or Agfa isochrome plates. The photographs were microphotometered as described, using the arc spectrum of iron as reference spectrum. Maxima were recorded from the curves with an accuracy varying from ± 10 Å. in the regions of longer wave-length or broad bands to ± 1 or 2 Å. with short wave-lengths or narrow bands.

White *pyrene*, purified by chromatographic fractionation, in hexane or benzene solutions gave three clear bands at 3940, 4050 and 4187 Å. and then a zone of general fluorescence without any distinct structure, from 4250 to about 4700 Å. The intermediate compounds, β -1-pyrenoylpropionic acid and 4'-keto-1':2':3':4'-tetrahydro-1:2-benzpyrene gave different spectra in benzene solution from those in hexane solution. In Schroeter mixture [Hieger, 1930] the fractions boiling at 260–300° and above 300° are very similar. Bands for the fraction B.P. 260–300° are noted at 4065, 4100–4110, 4200–4220, 4300, about 4450, at 4555 and 4700–4750 Å. 1:2-Benzpyrene gave spectra which varied considerably with concentration and markedly too with solvent. In benzene solution at concentrations 0.002–0.02 g. l., lines with maxima at 4075, 4112, 4182, 4312, 4580 and 4880 Å. were observed. In stronger solution (0.2 g./l.) these lines merged to 3 broad bands at 4175, 4300 and 4750 Å., whilst fluorescence faded off about 4150 Å. and was extinct at 4100 Å.; in still stronger solution (2 g./l.) a band at 5300 Å. appeared. Similar tendencies were revealed in hexane solutions at concentrations 0.005, 0.05, 0.5 g./l. (Fig. 1). Such effects seemed characteristic

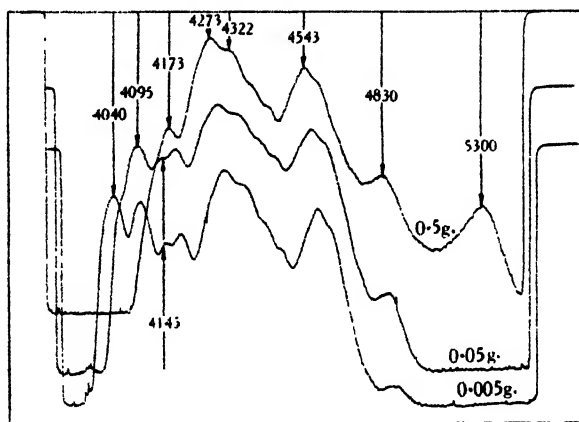


Fig. 1. Fluorescence of 1:2-benzpyrene in hexane solution. 0.5, 0.05 and 0.005 g. 1:2-benzpyrene per l. hexane.

both of our own synthetic material and of a similar sample kindly supplied by Dr J. W. Cook. In explanation, it seems likely (a) that some fluorescence bands are reabsorbed in strong solution (auto-absorption) and (b) that impurities may be present (as suggested by our data for ultra-violet absorption).

We find indeed that purification of 1:2-benzpyrene (m.p. 171–172° raised to 176–177°) has not only changed the shape of the absorption curve but shifted the maximum some 50 Å. towards longer wave-lengths (see, however, Mayneord and Roe [1936]). Even more striking effects of purifying 4'-keto-1':2':3':4'-tetrahydro-1:2-benzpyrene (m.p. 168–171° raised to 171.5–173.5°) were apparent in that the shape of the curve was markedly changed and band shifts of some 280 Å. (from 4540 to 4270 Å. and from 4740 to 4450 Å.) occurred.

In neither solution were those bands at 4000, 4180 and 4400 Å., which are specific for carcinogenic tars [Hieger, 1930; 1933], revealed.

It therefore seems improbable that fluorescence spectra characteristic of purified hydrocarbons would be recognisable in complex mixtures such as tar or Schroeter mixture. Even experimental conditions have a noticeable influence on the fluorescence optimum [Boutaric and Bouchard, 1933]. Moreover, it would only be possible to find constant bands in varying mixtures of fluorescent substances if the fluorescence power, Φ given by $\Phi = \Phi_0 e^{-Kc}$ [Perrin, 1924], and optimum concentration, c_m , were identical in all fluorescent substances in the mixture and in practice this is never the case. Winterstein *et al.* [1934] have demonstrated that the blue fluorescence of anthracene is almost completely extinguished by adding 1/100,000th part of naphthacene, and totally so with 1/30,000th part. Thus it seems impossible to ascertain the fate of carcinogenic hydrocarbons in tissue solely from fluorescence data. Indeed, so sensitive do the spectra seem to traces of contaminants that they constitute, rather, a delicate test for chemical purity.

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C. FLUORESCENCE SPECTRUM OF 1:2-BENZPYRENE.

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HIEGER [1930¹; Cook *et al.*, 1933] used the fluorescence spectra of various pitch fractions to aid in the isolation of 1:2-benzpyrene as the carcinogenic hydrocarbon contained therein. The low dispersion of his spectrograph permitted him to record only the three main bands. This work has therefore been repeated using a Hilger

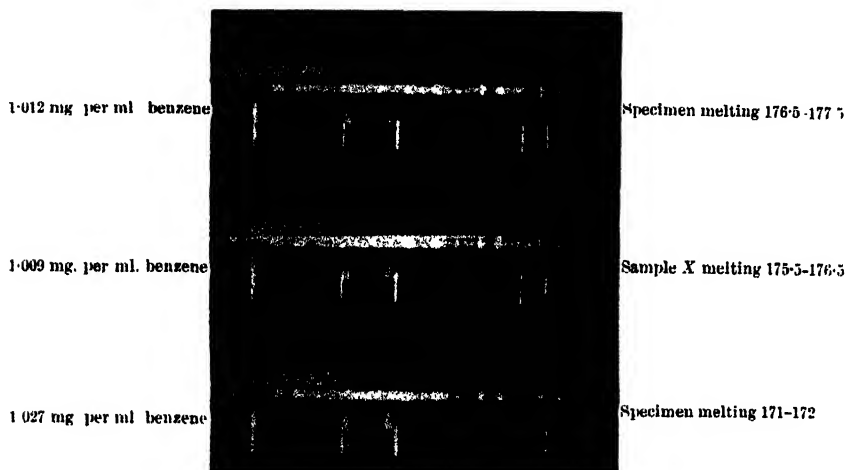


Fig. 1. 1:2-Benzpyrene solutions (Cook's samples).
Exposure 1 hour (Panchromatic plate).

medium quartz spectrograph (dispersion of about 40 Å./mm. at 4000 Å.) and light from a mercury arc filtered through Wood's glass. Synthetic samples of 1:2-benzpyrene (from Prof. Cook and from Prof. Sannié) gave by visual observation of the photographic plates apparently identical results. A benzene solution of 1 mg./10 l. showed three main bands with short wave-length edges at approximately 4040, 4270 and 4540 Å. (Hieger's 4000, 4180 and 4400 Å. bands). These were seen to be multiple bands, and from a visual estimation the position of the maxima were at 4055, 4110, 4180, 4310, 4580, 4880, 5500 Å. There is thus good agreement with the microphotometered determinations of Sannié [1936] except for the weak band at 5500 Å. (Sannié, 5300 Å.).

We do not agree, however, that the effect of impurities on the fluorescence spectrum of 1:2-benzpyrene is as great as suggested by Sannié [1936]. Three samples of benzpyrene (1) isolated from pitch (Cook's sample X, m.p. 175.5-176.5°), (2) synthetic (Cook's pure sample, m.p. 176.5-177.5°) and (3) crude

¹ The 3680 Å. mercury line mentioned is really the strong 3650 Å. line.

synthetic (Cook's sample, M.P. 171–172°) gave under comparable conditions (1 mg./ml. benzene, 1 hour's exposure to U.V., identical Panchromatic plate) fluorescence bands which appear by visual estimation to be in the same positions (Fig. 1). There was no shift to longer wave-lengths of 50 Å. with elevation of the M.P. from 171–172° to 176.5–177.5°, and such a shift would be easily visible with the instrument used. Furthermore the addition of 1:2-benzanthracene to benzpyrene solution to the extent of 30 % by weight seems to have no effect on the positions of the band edges. It appears therefore from these experiments that the fluorescence spectrum can still be employed as a useful qualitative test for the presence of 1:2-benzpyrene.

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CI. TUMOUR METABOLISM IN THE PRESENCE OF ANTI-CARCINOGENIC SUBSTANCES.

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(Received February 14th, 1936.)

THE discovery by Warburg of the characteristic glycolytic metabolism of malignant tumours has been amply confirmed. Nevertheless, little evidence has been found supporting his claim [Warburg, 1930] that the origin of tumours may be referred to the development of a non-oxidative type of metabolism in tissues partially asphyxiated by morbid processes. Orr [1934; 1935] showed that interference with the local circulation accelerated carcinogenesis due to tar and dibenzanthracene and suggested that Warburg's hypothesis might explain such results. It occurred to us that the efficacy of the anti-carcinogenic substances of Berenblum [1929; 1935] might be due to some ability to inhibit glycolysis in cells which were developing the tumour type of metabolism.

Of these anti-carcinogenic substances, mustard gas ($\beta\beta'$ -dichlorodiethylsulphide) has been most fully investigated [Berenblum, 1929; 1931]. A number of chemically related compounds have been similarly tested on tarred mice, as well as certain irritants which, while chemically dissimilar, produce similar anatomical changes on application to mouse skin [Berenblum, 1935]. Of the former group, the irritant members inhibited tumour formation and the others did not, while of the latter group only cantharidin had an effect comparable with that of mustard gas. We have now examined the effect of certain of these substances on tissue metabolism.

METHODS.

The chief difficulty in examining the metabolism of tissues by the manometric method in presence of mustard gas depends on the instability of this compound. At 37° in slightly alkaline solution (bicarbonate Ringer) mustard gas is rapidly hydrolysed with liberation of HCl, and the resultant p_H change may be greater than is permissible in metabolism experiments. This behaviour of mustard gas, as well as its low solubility in water, precluded the use of the usual tissue slice technique. Mustard gas however has a high lipid-water partition coefficient, and we hoped by adding it directly to the tissue to impregnate the tissue lipids with it and thereby to delay its hydrolysis. It was found that if a drop (roughly 25 mg.) of mustard gas were distributed through 4-5 g. of tissue mince by further mincing with fine scissors, the mustard gas incorporated in the mince had then only a very slight effect on the p_H of the bicarbonate Ringer. For this reason minced tissue was employed in the experiments here described. Manometer readings were begun about 30 min. after the addition of mustard gas.

In our experience the metabolisms of minced and sliced tumour (JRS) tissue differ only quantitatively. Table I gives the mean values obtained for the

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Table I. *Metabolism of minced and sliced tumour tissue.*

		Q_{O_2}	$Q_{CO_2}^{(a)}$	$Q_{CO_2}^{(N)}$
Mince	Mean of 20 experiments	3.7	9.8	—
Mince	Mean of 3 experiments	3.4	9.1	14.5
Slices	Mean of 5 experiments	8.6	23.2	36.3*

* Mean of 2 experiments only.

respiration (Q_{O_2}), the aerobic glycolysis ($Q_{CO_2}^{(a)}$) and the anaerobic glycolysis ($Q_{CO_2}^{(N)}$) of Jensen sarcoma mince and slices. They refer to periods of 40 min. duration at 37°. The figures for the metabolism of slices accord with those of Crabtree [1934], who showed further that the respiration of JRS in phosphate Ringer is 20–30 % lower than that in bicarbonate Ringer. Although the metabolism of the mince is considerably lower than that of the slices, the ratio $Q_{O_2}:Q_{CO_2}^{(a)}:Q_{CO_2}^{(N)}$ is almost the same, *i.e.* 1:2.7:4.3 for the mince and 1:2.7:4.2 for the slices. The mechanical injury to the tissue in mincing has therefore depressed proportionately the oxidative and glycolytic metabolisms. Many workers have found that freezing and thawing destroys the glycolytic power of tumour tissue, and similarly attempts to prepare glycolytically active cell-free extracts resembling the active muscle extracts of Meyerhof have consistently failed. These facts, together with the figures for mince and slice metabolism given above, suggest that glycolysis and respiration in tumour tissue are equally dependent upon the integrity of the living cell. The measured metabolism of tumour mince is therefore probably confined to the intact cells, and the relation between the respiratory and glycolytic phenomena in mince is presumably no less normal than in slices.

The Jensen sarcoma tissue was taken from rats immediately after the animals had been killed by a blow on the head. Only peripheral tissue, as free as possible from necrosis, was selected. The tissue was minced with fine curved scissors and the metabolism measured according to the principles established by Warburg. Barcroft differential manometers were used, 0.25 ml. of mince being suspended in 4.75 ml. of phosphate saline for respiration measurements and in the same volume of bicarbonate Ringer containing 0.2 % glucose for the measurement of glycolysis. In the latter case the compensating vessel of the manometer contained 0.25 ml. of the same mince in 4.75 ml. of bicarbonate Ringer without glucose. The phosphate Ringer was made up from isotonic solutions of NaCl, KCl, $CaCl_2$, phosphate (p_H 7.4) and 10 % glucose in the proportions 50:1:1:20:1.5; the bicarbonate Ringer consisted of isotonic solutions of NaCl, KCl, $CaCl_2$, $NaHCO_3$ in the proportions 50:1.2:1.2:10. The manometers were shaken in a thermostat at 37° and were filled three times, after evacuation at the pump, with O_2 , $O_2 + 5\%$ CO_2 or $N_2 + 5\%$ CO_2 .

$\beta\beta'$ -Dichlorodiethylsulphide, ethylene-bis- β -chloroethylsulphide and cantharidin are also hydrolysed in bicarbonate Ringer at 37°. The sulphone and ethylene compound form hydroxy-derivatives with liberation of free HCl, and the cantharidin is converted into cantharidic acid, of which it is an anhydride (formula, Table IV). The rate of acid production from these substances under the conditions of manometric metabolism experiments was determined. 5 ml. of bicarbonate Ringer were placed in each vessel of the Barcroft apparatus, and shaken with $O_2 + 5\%$ CO_2 at 37° until equilibrium was attained. The material under test was then tipped in from a Keilin tube as usual and the course of hydrolysis followed by observation of the CO_2 displacement from the bicarbonate. Fig. 1 shows that the sulphoxide is not hydrolysed, and that the rates of liberation of acid from the sulphone, ethylene-bis-chloroethylsulphide and

cantharidin are mutually similar but less than from mustard gas. Fig. 2 however suggests that the rate of acid liberation from ethylene-bis-chloroethylsulphide and from cantharidin is limited by the low solubility of the compounds since smaller amounts are hydrolysed at greater speeds relative to the sulphone.

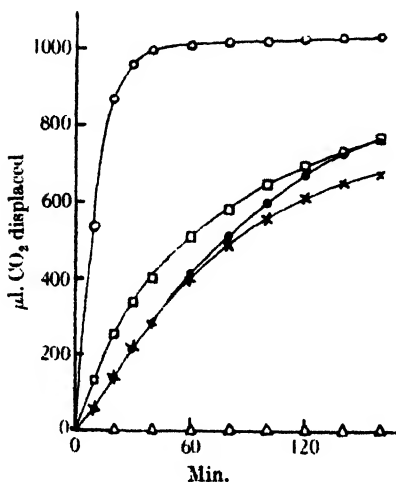


Fig. 1.

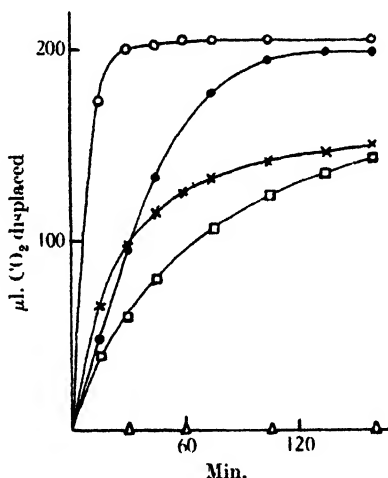


Fig. 2.

Fig. 1. Hydrolysis of mustard gas *etc.* in 5 ml. bicarbonate Ringer at 37°.

- Mustard gas, 4.1 mg.
- $\beta\beta'$ -Dichlorodiethylsulphone, 4.7 mg.
- △ $\beta\beta'$ -Dichlorodiethylsulphoxide, 5.2 mg.
- × Ethylene-bis- β -chloroethylsulphide, 4.8 mg.
- Cantharidin, 4.7 mg.

Fig. 2. As Fig. 1 but 1.0 mg. of each substance.

Since these substances are solid at ordinary temperatures it was not desirable to apply them to the mince as in the case of mustard gas. They were added in the form of 5 mg. of a fine powder directly to the medium in the Barcroft vessels in both sides of the manometer.

At the end of many of the experiments the media were transferred in syringes to a glass electrode apparatus of the Stadie type in which the p_H values were measured. The small p_H changes were insufficient to play any part in the effects observed: this was checked in the case of cantharidin by special experiments. The chief danger of acid production, of course, lies in the measurement of glycolysis, for accuracy in this measurement depends on the precise balancing of CO_2 displacement from this cause on the two sides of the manometer. Therefore in a few experiments the manometric measurement of glycolysis was confirmed by chemical estimation of the lactic acid formed, using the method of Friedemann, Cotonio and Shaffer [1927].

RESULTS.

The respiration (O_2 absorption) and glycolysis (CO_2 displacement) of 0.25 mg. of minced tumour tissue with and without impregnation by mustard gas are shown in Table II. The marked inhibition of glycolysis rather than respiration was confirmed in respect of aerobic glycolysis by chemical estimation of the lactic acid formed at the end of the period of manometric observation (Table III). The effects of the other irritants and poisons tested on the metabolism of minced Jensen sarcoma are shown in Table IV.

Table II. *Effect of mustard gas on the metabolism of minced tumour tissue.*

Duration of experiment, 40 min.									
Tissue	Respiration (μ l. O ₂)		Aerobic glycolysis (μ l. CO ₂)		Anaerobic glycolysis (μ l. CO ₂)		Percentage inhibition by mustard gas		
	Control	Exp.	Control	Exp.	Control	Exp.	Respiration	Aerobic glycolysis	Anaerobic glycolysis
JRS*	99	66	274	54	--	--	33	80	--
JRS	118	83	415	67	--	--	30	84	--
JRS	117	70	338	78	--	--	40	77	--
JRS	131	104	358	115	--	--	21	65	--
JRS	107	94	--	--	305	106	12	--	65
JRS	119	91	--	--	446	129	24	--	71
							Av. 27	76	68
M 63†	67	57	111	29	--	--	15	74	--

* JRS = Jensen rat sarcoma.

† A transplantable mouse carcinoma kindly supplied by the Imperial Cancer Research Fund Laboratories.

Table III.

Duration of experiment, 1 hour. Figures in brackets represent lactic acid equivalent to CO₂ displaced.

Aerobic glycolysis					Percentage inhibition			
Respiration		Manometric (μ l. CO ₂)		Chemical (mg. lactic acid)		Glycolysis		
Control	Exp.	Control	Exp.	Control	Exp.	Respiration	Manom.	Chemical
163	150	538 (2.15)	172 (0.69)	1.98	0.58	8	68	71
166	135	369 (1.48)	150 (0.60)	1.48	0.54	19	59	61

Table IV.

Compound	Structure	Respiration (μ l. O ₂)		Aerobic glycolysis (μ l. CO ₂)		Percentage inhibition	
		Control	Exp.	Control	Exp.	Respiration	Glycolysis
Mustard gas	S(CH ₂ .CH ₂ Cl) ₂	—	—	—	—	27*	76*
Thiodiglycol	S(CH ₂ .CH ₂ OH) ₂	171	153	490	510	11	— 4
		112	103	318	266	8	16
$\beta\beta'$ -Dichlorodiethyl- sulphoxide	OS(CH ₂ .CH ₂ Cl) ₂	112	80	318	273	29	14
		130	102	372	253	22	32
$\beta\beta'$ -Dichlorodiethyl- sulphone	O ₂ S(CH ₂ .CH ₂ Cl) ₂	121	61	399	68	50	83
		130	86	372	47	34	87
		88	42	268	41	52	85
Ethylene-bis- β - chloroethylsulphide	C ₂ H ₄ (S.CH ₂ .CH ₂ Cl) ₂	136	96	331	260	29	21
		177	150	390	374	15	4
Cantharidin		116	100	368	211	14	43
		131	90	329	115	31	65
		110	73	309	80	34	74
		139	114	324	125	18	61
		132	107	408	211	19	48
Sodium fluoride†		135	132	311	145	2	53
M/500		131	122	320	145	7	56
Acridine 1/1000		116	87	368	416	25	— 13
		135	94	311	381	30	— 22
Croton oil		110	120	309	321	— 9	— 4
		92	134	257	302	— 46	— 18

* Average figures (Table II).

† Ca-free Ringer used in this case.

To ensure that increased acidity of the medium due to hydrolysis was in no way responsible for the inhibitions of glycolysis observed, experiments were carried out in the case of cantharidin in which the acid production from this cause in the experimental vessel was compensated by occasional addition of suitable amounts of alkali (Table V). 0.5 ml. of minced tumour was suspended

Table V.

Time	Additions to	
	Flask A	Flask B
4.00	25 ml. bicarbonate Ringer	25 ml. bicarbonate Ringer
4.07	0.5 ml. JRS mince	0.5 ml. JRS mince
4.09	—	25 mg. cantharidin
4.11	0.5 ml. 10% glucose	0.5 ml. 10% glucose
4.24	0.45 ml. isotonic NaCl	0.45 ml. N/20 NaOH (made isotonic with NaCl)
4.39	0.50 " "	0.50 " "
4.54	0.45 " "	0.45 " "
5.09	0.40 " "	0.40 " "
5.11	Experiment stopped. p_H and lactic acid estimated	
Final p_H	7.30	7.23
Total lactic acid (mg.)	3.72	2.29
Initial lactic acid in mince (mg.)	0.88	0.88
Lactic acid formed (mg.)	2.84	1.41
Inhibition	—	50%

with and without 25 mg. powdered cantharidin, in 25 ml. of glucose-bicarbonate Ringer in 100 ml. conical flasks through which $O_2 + 5\% CO_2$ was passed. The flasks were shaken in the thermostat at 37° . The amount of alkali required was calculated from Fig. 1. Lactic acid formation was determined chemically. In three such experiments the inhibition of aerobic glycolysis was 50, 47 and 50% respectively.

NaF in $M/500$ concentration had only a slight effect on the respiration of JRS mince, but diminished the glycolysis to about half its normal magnitude. Acriflavine (1/1000) had a marked inhibiting action on the respiration whilst increasing the aerobic glycolysis by 10–20%. Croton oil, which is a powerful skin irritant, was unique in that it appeared to stimulate both respiration and glycolysis.

DISCUSSION.

Of the compounds examined in the foregoing section, sodium fluoride and acriflavine have not been tested for anticarcinogenic properties. They were investigated in order to satisfy us that tissue mince under their influence behaved in a manner comparable with that of slices. The results were satisfactory in that NaF exerted a selective action on glycolysis similar to that reported by Crabtree and Cramer [1934] for slices, whilst the behaviour of mince in the presence of acriflavine did not differ from that reported by Mellanby [1933] in the case of slices.

Inhibition of glycolysis to a much greater extent than of respiration was observed with mustard gas, dichlorodiethylsulphone and cantharidin, but not with thiodiglycol, dichlorodiethylsulphoxide or croton oil. The first three are known to inhibit the induction of tar tumours in mice; the others do not produce this effect. This suggested that the capacity for inhibiting glycolysis much more markedly than respiration might be a general characteristic of anticarcinogenic compounds. The behaviour of ethylene-bis- β -chloroethylsulphide, however, does not accord with such a view. This compound is quite as potent

as mustard gas in preventing tar tumours in mice, but its inhibiting effect on glycolysis was rather less than that on respiration. It may, however, be pointed out that the conditions obtaining when it acts in the one case on mouse's skin and in the other on tumour mince are widely different. If the ultimate agent in anti-carcinogenesis were a slowly formed reaction product of ethylene-bis- β -chloroethylsulphide, then the absence of any effect during the short period of the metabolism experiments is understandable.

In a few experiments the effects of mustard gas and of cantharidin on the metabolism of rabbit brain and chick embryo, tissues with a characteristically high anaerobic glycolysis, were examined. In each case the inhibition of anaerobic glycolysis was of the same order as that found for Jensen sarcoma tissue. The respiration of these tissues, however, seemed more sensitive to the irritants than was that of tumour tissue, and this was especially true of embryo, in which the percentage inhibition of respiration was about the same as that of glycolysis.

The depression of respiration of Jensen sarcoma by mustard gas *etc.* increased progressively during the course of an experiment, whilst a simultaneous increase in the inhibition of glycolysis was less marked. The major effect was at first on glycolysis, but the tissue gradually approached a condition in which respiration and glycolysis were almost equally depressed. It is difficult to assess the significance of this. Crabtree and Cramer [1934] suggested that the inhibition of respiration by iodoacetate is an indirect effect accompanying the diminished glycolysis, and the same may hold for the effect with which we are here concerned. On the other hand, the inhibition of glycolysis by mustard gas observed in the later stages of an experiment may be smaller than the true effect of the substance, on account of the considerable decrease in glycolysis of the control tissue, which is largely due, we suppose, to the accumulation of lactic acid and other metabolites.

During the progress of this investigation Jány and Sellei [1935] published a report on the effects of a number of "poison gases" on the metabolism of *B. coli*. It was found that mustard gas inhibited glycolysis by *B. coli* (aerobic glycolysis by 64 %) but that respiration was more than doubled. Similarly the respiration of Ehrlich rat carcinoma was stimulated by 164 % and the aerobic glycolysis inhibited by 94 %. Whilst this inhibition of glycolysis resembles our results with Jensen sarcoma, we have never seen any stimulation of respiration in the latter tissue. Jány and Sellei measured both respiration and glycolysis in bicarbonate Ringer, and appear to have been unaware of the rapidity of hydrolysis of mustard gas in this medium; this may have introduced errors into their results.

SUMMARY.

$\beta\beta'$ -Dichlorodiethylsulphide (mustard gas), $\beta\beta'$ -dichlorodiethylsulphone and cantharidin, which inhibit the induction *in vivo* of tar tumours, reduce the glycolysis of minced Jensen sarcoma tissue *in vitro* more than its respiration.

$\beta\beta'$ -Dichlorodiethylsulphoxide, thiodiglycol and croton oil, which have no inhibiting effect on carcinogenesis, do not reduce glycolysis more than respiration.

Ethylene-bis- β -chloroethylsulphide, which inhibits the induction of tumours, has no selective action on glycolysis.

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CII. AN ABSORPTION APPARATUS FOR THE MICRO-DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

V. THE MICRO-DETERMINATION OF BROMIDE, WITH APPLICATION TO BLOOD AND URINE AND OBSERVATIONS ON THE NORMAL HUMAN SUBJECT.

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INTRODUCTION.

A METHOD has been described [Conway, 1935] for the micro-determination of chloride depending on the oxidation of chloride to chlorine by an acid permanganate mixture in the outer chamber of an absorbing "Unit" [Conway and Byrne, 1933]. The chlorine formed is estimated iodimetrically, the whole procedure being conducted at room temperature. Quantitative determinations could be carried out with as little as 0.5 γ Cl, which contained in 2 ml. represents 0.025 mg./100 ml. Cl. Still smaller quantities could be determined in apparatus of reduced size.

The principle and general technique of the chloride determination may also be applied to that of other halogens. Both free bromine and iodine are sufficiently volatile to require about the same absorption times as chlorine. In the present communication the micro-bromide technique is described, bromide being determined to an ultimate standard deviation of 0.08 γ Br, which in milli-equiv. is about one-third that found with chloride. The standard deviation of the blank value may be expressed as 0.037 γ Br, which is only about one-seventh of the equivalent value found with chloride. The difference probably arises from minute traces of chloride likely to be present in water and on glassware. These ultimate errors for chloride and bromide determinations appear to be the lowest yet recorded. The early papers on micro-determination of bromide have been reviewed by Bernhardt and Ucko [1925]. Accounts of the more recent developments may be found in the papers of Quastel and Yates [1934], Dixon [1935], Bertram [1933], Stoll and Brenken [1934], Indovina [1935], Leipert [1935] and others. Here reference will be made only to such previous work having immediate bearing on the present method and the results obtained for normal blood and urine.

Such micro-bromide determinations consist essentially of three stages. Firstly, the ashing of the material. Secondly, the oxidation of the bromide to bromine by a suitable oxidant. (These two stages may be combined in wet ashing methods such as that of Leipert and Watzlawek [1934].) Thirdly, the determination of the liberated bromine after extraction, aeration *etc.*

In the present method these three stages are also followed for blood and urine, ashing being obviously unnecessary for purely inorganic mixtures or such as contain only traces of organic matter (*e.g.* sea water). The stages are conducted

in a manner hitherto undescribed and permit the least absolute error of bromide with the least manipulation. Also, it is possible with this method to carry out large numbers of determinations at the same time.

The apparatus used for the liberation and absorption of the bromine is that already described in previous communications. In the outer chamber of the apparatus is contained a dichromate oxidation mixture and in the centre 1 ml. of 20 % KI in which the formed bromine is absorbed, liberating its equivalent of free iodine.

The ashing procedure. The ashing of the blood is carried out after preliminary removal of the proteins with methyl alcohol and subsequent evaporation of the cleared fluid. This reduces the incineration time to a few minutes and was introduced here for the reason that it is now practically certain that all the bromide in normal blood is present in inorganic form. If a complete ashing procedure is desired, the technique of Indovina [1935] seems very suitable. In this method magnesium oxide is employed to prevent bromide losses on heating.

The oxidation procedure. The oxidant found most suitable for bromide determinations is dichromate and sulphuric acid. The dichromate is used in powdered form and the sulphuric acid as 1 ml. of a solution containing 40 vol. % of sulphuric acid to 1 ml. of the ash extract. (Upwards of 48 vol. % may be used after bromide administration.) Acid dichromate of this strength has a scarcely detectable influence on 1 ml. of 1 % NaCl even after 20 hours' action, whereas all the bromide is oxidised and absorbed in 2-2½ hours.

The presence of iodide interferes with this method as shown in the experimental section. With normal blood and urine this is of little or no account as the final error introduced will not exceed 1 %.

After administration of iodide, the iodide present may be removed as iodine by treating the ashed extract in the outer chamber with dichromate in about 0.1N sulphuric acid, the inner chamber being empty. Raising the acidity to the required degree will then liberate the bromine which is absorbed and determined as usual. Instead of letting the formed iodine escape, it may be determined in a similar manner to bromide.

As referred to in a previous communication [Conway, 1935] the use of a suitable acid permanganate mixture would have the advantage of rendering the method independent of the presence of both chloride and iodide. It was found however to have the disadvantage of causing marked losses as bromate, when the concentration of bromide for determination is very small.

The determination of the bromine formed on oxidation. In the present method the bromine diffuses into the central chamber of the "unit", liberating its iodine equivalent from 20 % KI. The iodine is then determined as for chloride [Conway, 1935] by titrating with thiosulphate if more than 80 γ Br are present; an absolute colorimetric determination of the iodine colour itself is likewise found very suitable for the range from 80 to 16 γ Br. Below this level, starch is added and the colour produced is colorimetrically determined.

In the method used by Leipert and Watzlawek [1934] for the determination of the bromide content of normal blood the formed bromine is oxidised to bromate after absorption into alkali, the bromate being then determined iodimetrically. An advantage of this procedure is that six times the equivalent of the original bromine is finally present as iodine. This variation could also be used with the "unit", with possible advantage for the minutest bromide determinations.

The delicacy of the method selected however is so great that it is doubtful if a substantial increase in accuracy would result, and further operations with

numerous reagents would be required. Also errors in the bromine formation and absorption are likewise multiplied by six.

With the present method, using starch, each γ Br will in fact cause an increase in the extinction coefficient of the mixed fluid from the central chamber of 0.09 (for a wave-length of $464m\mu$ approximately).

PROCEDURE.

Inorganic.

(a) *Above 80 γ Br.* Into the outer chamber of a "unit" is placed a small quantity of pure powdered potassium dichromate—roughly about 0.2 g.—by means of a spoon spatula, and into the central chamber 1 ml. of 20 % KI. Into the outer chamber is now introduced 1 ml. of the bromide solution and the lid smeared with the fixative (see below) and placed in position. The unit is slightly tilted by resting it on the edge of a spare lid, the lid is slightly displaced and 1 ml. of a solution containing 40 % (by vol.) sulphuric acid is run in quickly without blowing—best from a simple tube with a mark at 1 ml. approximately. A solution containing 45 % acid (by vol.) may be used when the Br/Cl ratio exceeds 1/500. After the introduction of the acid the lid is quickly replaced. The fluids in the outer chamber are well mixed by rotation. After 2 hours the free iodine in the central chamber is titrated with 0.05*N* thiosulphate from the special burette [Conway, 1934], the number of divisions on the burette being multiplied by the bromine equivalent or factor which may be determined as for chloride by making some initial determinations with standards or titrating standard iodine in 20 % iodide in the central chamber. In this latter case the thiosulphate required is multiplied by 0.97 to allow for the slight constant loss of iodine from the central chamber during a determination.

(b) *Below 80 γ Br.* The procedure is carried out in the same way as for the previous method, except that the 1 ml. of iodide (which should have been carefully delivered) in the central chamber is examined colorimetrically. This is done either by direct colorimetric investigation without standards or by the addition of 0.2 % starch solution and subsequent determination.

Without standards (to about 16 γ Br). The contents of the central chamber are pipetted into a micro-colorimeter cup and the plunger set at 8.35 mm. or convenient multiple. Using the "grey solution" of Thiel [1933] and Leitz filter No. 3 the depth in mm. of grey solution multiplied by 10 gives the γ Br analysed. 1.4 γ are added to the result as representing a small constant loss as bromate. It is advisable that the depth of the fixed plunger be so chosen that the mm. grey solution required for a match do not much exceed 10 for this direct colorimetry, though this precaution is not necessary when using starch. For this absolute colorimetry the Pulfrich photometer may also be employed or the new Leifo instrument of Leitz, the extinction coefficient being given directly on the instrument and the bromide concentration being subsequently read from a graph or tables.

With starch addition (to about 2 γ Br). This method is most suitable for bromine quantities under 16 γ , but may be used to cover the whole range from 80 γ Br downwards. Where the amount is under 16 γ , 0.5 ml. of a 0.2 % starch solution is added to the central chamber but for the range between 80 and 16 γ , 2.0 ml. of the 0.2 % starch solution must be used. The mixed contents from the central chamber are pipetted into a micro-colorimeter cup and the iodine is estimated by comparing it with standard iodine in 20 % KI similarly treated,

or by absolute colorimetry. Using the grey solution the fixed plunger in the starch iodide solution is put at 29.0 mm. (or convenient multiple or submultiple) and the reading of the grey solution multiplied by 0.2 with subsequent addition of 1.4 gives the γ bromine analysed. Leitz filter No. 3 (464 $m\mu$) is used. (If 2 ml. starch have been added the reading is multiplied by 0.4.) The values obtained by absolute colorimetry may be checked by standard iodine and starch as described in the chloride method. It may be mentioned however that a greater constancy with absolute colorimetry in relation to the starch-iodide colour was obtained than was anticipated, provided that the same starch solution was used throughout, so that the whole procedure below 80 γ Br could be conducted without standards.

Ashing and extraction procedure for blood and urine.

Normal blood (1 ml.). Into a 15 ml. centrifuge-tube is pipetted 1 ml. of blood, 12 ml. of 95% methyl alcohol are added and the whole is well mixed. The tube is then capped and centrifuged for 5 min. 10 ml. of the clear fluid are pipetted into a small crucible (20–30 ml. capacity) and 0.2 ml. of a standard KBr solution containing 2 γ Br is added. The fluid is dried in the oven at 90–100° and then the crucible is heated in the Bunsen flame until the bottom and sides have been brought for about 1 min. to a dull red heat. It is then cooled and 0.5 ml. of distilled water is pipetted into the crucible, mixed and rotated around the walls. The solution is then pipetted into the outer chamber of a unit. Another 0.5 ml. water is added and the process repeated. The presence of some carbon particles does not interfere. The determination then follows the inorganic procedure (above outlined) for quantities under 16 γ Br. The 2 γ Br are added to bring the amount analysed on to the linear curve. A blank determination is carried out in an exactly similar manner including the small bromide addition. The calculation of bromide analysed is then made as above, the blank value being subtracted and the addition of 1.4 γ Br here omitted.

Blood after bromide administration (0.2 ml.). A similar procedure is adopted. 5 ml. of 95% methyl alcohol are used and 3 ml. of the clear fluid pipetted off for drying. No bromide need be added. The mode of determining the free iodine in the central chamber will depend on the order of magnitude of Br expected. It may be noted that the ingestion of 2 g. KBr by the human subject will produce about 16–32 γ Br in 0.2 ml. of whole blood.

The recovery of bromide from Folin-Wu filtrates is described below.

Urine. The same procedure for ashing and extraction may be adopted for normal urine as for urine after bromide administration. Since the bromide in urine very closely follows the chloride it is always advisable to carry out a preliminary chloride determination. The bromide concentration has in fact little significance apart from this chloride figure. Where the urine contains the average quantity or more of chloride, 2 ml. are used for ashing with correspondingly larger volumes with reduced chloride concentrations. 2 ml. urine are pipetted into a crucible and dried in the oven. The crucible with dried contents is now held over the Bunsen flame and cautiously incinerated. The contents char at first, then fumes are emitted, the residue finally melting as a rule when the bottom of the crucible turns red. At this stage the crucible is withdrawn from the flame and cooled. The whole procedure does not require more than a few minutes, and the bottom of the crucible is brought only for about 1 min. to a dull red heat. After cooling, 2 ml. of distilled water are added and mixed with the contents. After a short time 1 ml. is pipetted into the outer chamber of a "unit", the standard method being then followed. If it is found difficult to obtain 1 ml. free

from many charred particles, 2 ml. of extract are centrifuged in a small tube, which procedure may be followed as a routine. After bromide administration, the free iodine present in the central chamber can be titrated with the *N*/20 thiosulphate from the special burette.

Notes on method and solutions required.

No special care need be taken to free the analytical reagents used from bromide, since it is found that they contain none. But it is advisable to clean the units, after the usual cleaning, with a little alcohol and ether, subsequently rinsing them with distilled water. For the smallest bromide quantities the units should not be exposed to direct sunlight during the absorption and may be covered with a light cloth; under these conditions the blank values will show no alteration even after 24 hours or more. Another point that may be emphasised is that 0.5 ml. of 0.2 % starch is no longer sufficient when the bromide exceeds 20 γ . 2.0 ml. are then a convenient amount and will be sufficient for the whole range.

40 % sulphuric acid. This is made up as a volume concentration, 40 ml. of the purest sulphuric acid (sp.gr. 1.84) being made up to 100 ml. with water. A certain latitude (of a few % at most) is permissible in the actual concentration, but the purest sulphuric acid must be used. 45 vol. % is advisable when the Br/Cl ratio exceeds 1/500.

20 % potassium iodide. The purest reagent should be employed, and for the minutest bromide determinations it should be freshly prepared. When over 80 γ Br are being determined, a slight coloration is of no consequence as it will be allowed for in the blank.

0.2 % starch. This is best prepared as described for chloride determinations.

The fixative. This is also the same as for chloride.

0.02N iodine. This is made up as a stock solution from *N* or 0.1*N* iodine and stored in a brown bottle in the dark.

Standard bromide. 1.489 g. of pure powdered and dry KBr are dissolved in 1 litre of water. Then each ml. contains 1000 γ Br.

EXPERIMENTAL.

Determinations on standard bromide solutions.

Table I gives a summary of the results obtained in solutions containing from 800 to 2 γ Br. The accuracy for the higher quantities resembles that already found with chloride, namely 0.6 % as a coefficient of variation for 800 γ Br compared with 0.5 % for 355 γ Cl. For the lower quantities, 8–2 γ Br, the bromide determinations are more accurate owing to the smaller blank values. The bromide blank values in fact arise merely from the light absorption by the added starch solution and may be occasionally due to traces of free iodine contained in the iodide solution. It will be seen from the table that for the 16 determinations from 8 to 2 γ Br the maximum deviation was 0.21 γ Br. For 8 determinations of 2.0 γ Br the standard deviation was 0.08 γ . Fig. 1 shows the bromide values obtained (iodine equiv. multiplied by 1.03) plotted against the values added to outer chamber. Here the constant loss of 1.4 γ Br has not been added to the calculated results: the validity of this correction is however apparent from the figure. The line has been extrapolated beyond 2.0 γ Br to cut the abscissa at 1.4. Some determinations of 0.8 γ Br indicate that the full curve leads from somewhat under this point

Table I. *Determination of bromide in standard solutions.*

Bromide analysed γ Br	No. of analyses	Mean value of bromide recovery γ Br (including addition of 1.4 γ Br)	Extinction coeff. of fluid from central chamber minus blank	Standard deviation of the individual determination γ Br	Maximum deviation found γ Br	Method of determining the free iodine in central chamber
800.0	6	803.2	—	4.8 (0.6%)	15.0 (1.9%)	Titration
80.0	7	79.9	0.471	1.4 (1.8%)	4.0 (5.0%)	Direct colorimetry
16.0	3	15.7	1.228	—	0.34 (2.1%)	Colorimetry with starch
8.0	18	8.13	0.578	0.12 (1.5%)	0.38 (4.8%)	..
6.0	4	6.01	0.396	—	0.10 (1.7%)	..
4.0	4	3.99	0.223	—	0.21 (5.2%)	..
2.0	8	2.03	0.054	0.08 (4.0%)	0.16 (8.0%)	..
Blanks	11	—	0.017	0.037	0.09	..

The 11 blank determinations were carried out in the one series. A few obviously spoiled results have been omitted.

The method of calculating the results is described in the text.

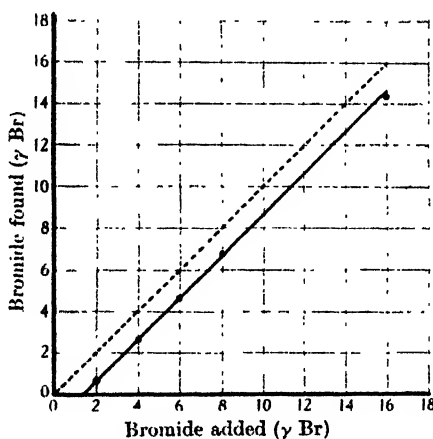


Fig. 1.

Fig. 1. — The bromide found (determined from the liberated iodine $\times 1.03$). - - - - The result obtained using the same colorimetric method with the theoretical equivalent of free iodine added immediately to 1 ml. of 20% KI. The calculation in the text allows for the factor 1.03 and the constant addition of 1.4 γ Br.

into the origin. The addition of a trace of bromide (2 γ Br per ml.) in the 40% sulphuric used for the determination and the blank will eliminate the addition of 1.4 γ Br to the result. The same result is achieved by adding 2 γ Br (as bromide) directly into the outer chamber of the units, the strength of the acid added being suitably altered for the extra volume.

The effect of chloride and iodide on the bromide determinations.

For biological determinations it is essential that chloride should have no appreciable influence since it will almost always be present in largely preponderating concentrations. With the above determinations in the 2 hours of bromide absorption the chloride oxidation is scarcely measurable when the chloride concentration in the outer chamber is of the order 0.5–1.0% NaCl (i.e. 1–2% in the fluid for examination). These represent the higher range of

concentration likely to be met with in urine analysis: even after 24 hours' action the amount represents less than 2γ Br. It may be noted however that when the chloride is increased to concentrations of the order of 10% the amount of chloride oxidised is increased in much greater proportion. This effect is shown in Table II. If 1 ml. of 10% NaCl was added to the outer chamber with 1 ml. acid,

Table II. *Effect of chloride on bromide determinations (dichromate oxidation).*

Experimental group	Chloride in outer chamber expressed as its bromine equivalent γ	Bromide in outer chamber γ Br	Time of action hours	Total halogen oxidised as Br equivalent (+ 1.4γ Br)
1	136,700	-	21	52
2	13,670	-	20	1.6 (maximum value)
3	13,670	-	2.5	- (indeterminable)
4	13,670	4	2.5	4.06

The free iodine in the central chamber was determined colorimetrically as described in the text. The average room temperature was 15° .

an excess value of about 6γ Br at room temperature of 15° was obtained for the $2\frac{1}{2}$ hours' absorption, the chloride present at the same time representing an equiv. of 136,700 γ Br.

The effect of iodide may be seen from Table III.

Table III. *Effect of iodide on bromide determinations (dichromate oxidation).*

Experimental group	Potassium iodide in outer chamber as Br equivalent γ	NaCl in outer chamber as Br equivalent γ	Bromide in outer chamber γ Br	Total halogen oxidised determined as Br equivalent
5	80	13,670	8.0	48.4
6	8	13,670	8.0	13.8
7	-	-	8.0	8.0

The outer chamber of the units contained besides the 1 ml. of 40% sulphuric acid 0.2 g. solid dichromate and 1 ml. of a halogen mixture in each experimental group. In group 7 only standard KBr solution was used.

About half the equivalent of the iodide present appears as free iodine in the central chamber, the remainder being converted into iodate at the concentration of acid used.

Table IV illustrates how iodide may be removed if present in appreciable quantity. In the outer chamber were placed some solid dichromate, 1 ml. of

Table IV. *Removal of iodide before the bromide determination (dichromate oxidation).*

Into the outer chambers of a number of units were placed 0.2 g. (approx.) dichromate, 1 ml. of $N/1000$ KI and 0.1 ml. of NH_4SO_4 . The units were then exposed to the air with empty central chambers for varying periods, at the end of which 1 ml. of 20% KI was run into the central chamber and the unit covered by a lid with fixative, the iodine absorption being allowed to proceed for 2 hours at room temperature.

Time of exposure min.	Iodine subsequently absorbed in central chamber γ iodine	Time of exposure min.	Iodine subsequently absorbed in central chamber γ iodine
0	118	36	2.6
2	98	42	1.5
8.5	44	72	0.1
14.5	15	72	0.0
23	11	78	0.0

$N/1000$ KI (equivalent to 80γ Br) and 0.1 ml. N H_2SO_4 . The iodide is converted into free iodine, which entirely disappears in about 1 hour's exposure. Instead of permitting the iodine to escape, it may be determined in a similar manner to the other halogens. With an acidity of $N/10$ H_2SO_4 in the presence of dichromate, no appreciable fraction is converted into iodate.

Recovery of bromide added to shed blood. To 10 ml. of defibrinated blood was added 1 ml. of a standard bromide solution, 1 ml. of water being added to a second 10 ml. blood. A series of 0.2 ml. samples from both mixtures was then analysed. The results are shown in Table V, where the results from the blood

Table V. Bromide "recoveries" from blood.

Bromide added per 0.2 ml. blood γ Br	Blood analysed ml.	No. of analyses	Bromide recovered γ Br	Coefficient of variation of individual determination	Maximum deviation %
145	0.2	10	146	3.3	5.2
14.5	0.2	12	14.6	3.8	6.6

The blood for analysis was prepared by adding 1 ml. of standard KBr solution to 10 ml. o. defibrinated sheep's blood. For the blank values water was substituted for the standard KBr.

containing no added bromide were treated as blank values. Where 145γ added Br were contained in each 0.2 ml., 146γ were recovered as the mean of 10 determinations, a maximum error of 5.2% being observed for the individual analysis. When 14.5γ of added Br were present, 14.6 were recovered as the mean of 12 determinations, the maximum error being 6.6% of the mean.

The bromide recovery from blood is therefore quantitative.

Recovery of bromide added to urine. Some 2 ml. samples of normal urine were evaporated and incinerated as above. To other similar samples of the same urine 1 ml. of standard KBr solution was added, the whole evaporated and incinerated as before. The bromide was then determined, the urine containing no added bromide being used as a blank. The free iodine in the central chamber was determined by titration. The results are shown in Table VI. The recovery of added

Table VI. Bromide "recoveries" from urine.

Bromide added to crucible per 1 ml. urine γ Br	Vol. of urine incinerated ml.	No. of analyses	Bromide recovered γ Br	Maximum deviation found %
80	2.0	3	78	4.8
200	2.0	9	199	6.1 (second highest = 3.1)
400	2.0	3	392	3.3

2 ml. of distilled water were used to extract the incinerated urine, 1 ml. of the extract being taken for subsequent analysis.

quantities varying from 80 to 400γ Br was investigated. For the 15 results summarised in the table, 99% was recovered as a mean value. Only 1 of the 15 results showed a deviation of more than 4.8% from the mean.

The bromide content of normal blood and urine.

Normal whole blood and urine samples from 8 students were investigated in the manner described. The mean value for blood was 372γ Br/ 100 ml. individual values varying between 227 and 572 . The results are given in Table VII. The corresponding urine samples gave a mean value of 656γ Br/ 100 ml. with a range of variation of 297 – 855 . The chloride of the urine samples was determined as well as

Table VII. *Bromide in normal blood and urine.*

Subject	Blood bromide		Urine bromide		Urine chloride		Cl/Br (milli- equiv.) in urine
	γ Br per 100 ml.	Milli- equiv. per 100 ml. $\times 10^3$	γ Br per 100 ml.	Milli- equiv. per 100 ml. $\times 10^3$	mg. Cl per 100 ml.	Milli- equiv. per 100 ml.	
Sh.	360	4.50	705	8.81	501	14.1	1600
Cos.	257	3.21	297	3.71	355	10.0	2700
To.	227	2.84	685	8.56	778	22.2	2590
Cu.	341	4.26	550	6.86	611	17.2	2510
Mu.	572	7.15	702	8.78	688	19.4	2210
Ma.	487	6.08	757	9.46	599	16.9	1790
Con.	455	5.68	700	8.75	593	16.7	1910
Cr.	276	3.45	855	10.69	716	20.2	1890
(Fo.)	(1890)	(23.6)	(2450)	(30.6)			

The above figures for normal blood and urine were obtained on blood and urine samples from 9 students. For the last of the group it was found on subsequent inquiry that a course of bromide had been prescribed (amounting altogether to approximately 10 g. KBr) the last dose having been taken 7 weeks before the date of blood determination. The student was unaware that he had taken bromide.

the bromide, using the method described in a previous communication [Conway, 1935]. The ratio of chlorine to bromine, each expressed as milli-equiv. per 100 ml. is 2150 as an average value, individual values varying between 1600 and 2590. The chloride in the blood samples was not determined. If we assume that the mean ratio of bromine in the whole blood to bromine in the plasma is 1:1.12—a figure derived from Leipert's data [1935]—and that the concentration of chloride in plasma is rather constant and 10.35 milli-equiv./100 ml., then we may derive the mean value of Cl/Br for the plasma as 2180. Comparing this value with the corresponding ratio of 2150 for urine it appears that compared with chloride the bromide in urine is almost identical with that in blood. Leipert obtained a comparatively high ratio, namely 1:1.5.

Recovery of bromide from a Folin-Wu filtrate.

In the preparation of a blood filtrate from defibrinated blood prepared according to Folin and Wu [1919] standard bromide has been added to a final concentration of *ca.* 0.001*N*. A similar filtrate was prepared without added bromide. 1 ml. samples were taken in the outer chamber containing solid dichromate and the method carried out as previously described, the filtrate without added bromide serving as a blank. The free iodine in the central chamber was determined by direct colorimetry using the "grey solution" of Thiel and filter No. 3 (Leitz). The results illustrated in Table VIII show a

Table VIII. *Bromide "recoveries" from a tungstate filtrate.*

No. of determination	Added bromide in 1 ml. filtrate	Bromide recovered from 1 ml. filtrate
	γ Br	γ Br
1	78.9	79.0
2	78.9	82.3
3	78.9	79.0

quantitative recovery. In this determination of bromide in a tungstate filtrate it is better to add 1 ml. of a solution containing 45% sulphuric acid (by vol.) instead of the usual 40%. The use of the Folin-Wu filtrate is suitable only after bromide administration corresponding to a few g. or more in the human subject. Somewhat reduced "recoveries" were noticed in some experiments in which the filtrate contained only 8 γ Br in ml.

The effect of varying the conditions in the above bromide method.

Varying the acid concentration. The effect is illustrated in Fig. 2, the rate of absorption from bromine water being also given for comparison (dotted line). It will be seen that changing the acid concentration has a marked effect of a kind similar to that shown for chloride. 20 min. after adding the 40% sulphuric acid (as described above) 50% of the bromide has been oxidised and absorbed. When 20% acid was added only 4–5% of the bromide was recovered in the same time. In a similar way to the action of the permanganate system on chloride, a marked deficiency appears in the total amount recovered when the concentration of acid exceeds a certain limit (see curve *C*, Fig. 2). This deficiency

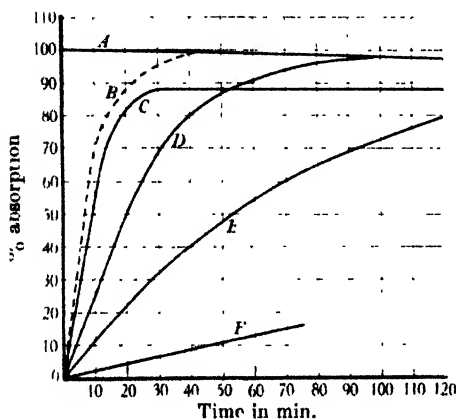


Fig. 2.

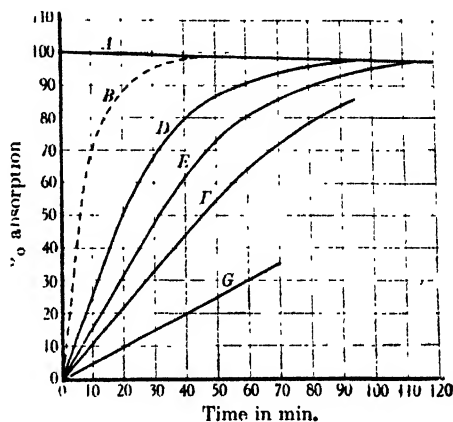


Fig. 3.

Fig. 2. Curves *C*, *D*, *E* and *F* represent the absorption rates of the bromine from the outer chamber containing 0.1 ml. of 0.1*N* KBr, 1 ml. of saturated dichromate and 1 ml. of sulphuric acid of strengths 75, 40, 30 and 20% (by vol.) respectively. Curve *A* represents the fall in percentage value of free iodine in the outer chamber introduced before a blank determination (allowance being made for blank values). Curve *B* represents the absorption of bromine from 2.1 ml. bromine water in the outer chamber.

Fig. 3. Curves *D*, *E*, *F* and *G* represent the bromine absorption from the outer chamber containing 0.1 ml. *N* KBr, 1 ml. of 40% (by vol.) of sulphuric acid and 1 ml. of saturated, half-quarter- and one-sixteenth-saturated dichromate respectively. Experiments conducted at room temperature of about 18°.

however is not evident until the concentration of acid in the mixed fluids exceeds 24%. The concentration of acid should in fact be maintained within 24–19%, since a higher concentration than 24% causes a loss in the total amount recovered and less than 19% gives an unduly slow absorption.

In Fig. 2, curve *A* shows that a slight constant loss of iodine occurs from the central chamber (approximately 1% per hour of the total amount present).

The effect of changing the dichromate concentration in the outer chamber. The effect is much less than changing the concentration of acid. Similar results were obtained with the permanganate system and chloride; but whereas the rate of chloride oxidation seemed to be proportional to the permanganate concentration, the influence of varying the concentration of dichromate appears to follow a square root relation.

The effect is shown in Fig. 3. It may be noted that saturated potassium dichromate at room temperature (18°) contains 9.8 g. dichromate per 100 ml. solution.

With regard to changes in the other conditions such as fluid volume, temperature *etc.*, the reader is referred to the previous communication on chloride [Conway, 1935].

DISCUSSION.

This method for micro-bromide determination is applicable to general inorganic analysis and to blood and urine determinations *etc.* With blood the normal bromide concentration may be determined in 1 ml. After bromide administration finger blood (0.2 ml.) can be used.

Pincussen and Roman [1929], supported by Zondek and Bier [1931] advanced the theory that normal blood bromide consisted largely of bromide combined in organic linkage and functioning like thyroxine as an important catalyst in the organism. The pituitary was thought to be the site of the formation of this substance. Its passage from the pituitary into the mid-brain was believed to be associated with the advent of normal sleep and the content of bromide in the blood to be much reduced in maniacal depressive states. The views of Pincussen and Zondek were based however on faulty methods as shown by Fleischacker and Scheiderer [1932].

Ucko [1934], who with Bernhardt [Bernhardt and Ucko, 1925: 1926] had obtained very high bromine values for the pituitary, has now stated that using a revised method he could not find more bromide in the pituitary than in other glands and that no valid evidence exists for assuming the presence of an organic bromide catalyst or for the assumption that a gland is associated with its production. From the work of Quastel and Yates [1934], Dixon [1935] and in particular the recent detailed studies of Leipert [1935], and of Leipert and Watzlawek [1935] there are no valid grounds for supposing that the normal blood bromide is other than the inorganic substance. Leipert has shown that protein precipitation, dialysis or ultrafiltration gives no indication of a bromo-protein compound. Moreover the bromine of blood is completely precipitated by silver nitrate, whereas organic bromide compounds of the type of dibromotyrosine are not precipitated by this reagent.

Concerning the origin of the normal blood bromide the greater part of this must come from the bromide associated with the natural chloride of the food although this does not often exceed 20% of the total ingested. The Br/Cl ratio in commercial chloride has been found here not to exceed 1:7000 for four samples, whereas the Br/Cl ratio of sea water and of ordinary drinking water (*e.g.* Dublin Vartry water) is of the order 1:700 to 1000. Presumably the natural foodstuffs possess a similar ratio to the latter with regional variations. The commercial chloride taken would therefore dilute the "natural" ratio—the general limiting value of which may be presumed to be that of the sea.

The failure of the evidence hitherto presented for an organic bromide compound in the blood by no means removes biological interest from micro-determinations of bromide. Among other considerations the study of the Br/Cl ratio may throw considerable light on the chloride metabolism as well as on the functioning of such organs as the kidney. From the clinical side also an easy and accurate method for carrying out micro-bromide determinations on very small quantities (finger blood after a single therapeutic dose) is of considerable interest.

SUMMARY.

1. A method is given for the micro-determination of bromide, using the special absorbing apparatus already described. With the method, bromide can be determined to an ultimate standard deviation of 0.08% Br.

2. Using standard KBr solutions, the coefficient of variation for a single determination of 800 γ Br was found to be 0.6% and with 80, 8 and 2 γ Br it was found to be 1.8, 1.5 and 4.0% respectively.

3. In the method the bromide ion is oxidised to free bromine in the outer chamber and the formed bromine is then absorbed in 1 ml. of 20% KI in the central chamber, the whole procedure being conducted at room temperature. The free iodine liberated is titrated with 0.05N thiosulphate (using the special burette already described) when the bromide exceeds about 80 γ . It is colorimetrically determined below this level, either directly or after starch addition, without standards, using modern methods of absolute colorimetry and in a similar manner to that already described for minute chloride determinations.

The determination may be also carried out with an ordinary colorimeter, after starch addition and using standards.

4. The oxidant selected as most suitable is acid dichromate. For all biological determinations where the chloride strength does not much exceed 1% as NaCl, the determination is entirely independent of the presence of chloride even when the molar concentration of chloride is 7000 times that of bromide. With acid dichromate the method is not independent of the presence of iodide, but iodide can be easily and completely removed by a preliminary treatment which may be also made the basis of an initial iodide determination.

5. Applications of the method to the determination of bromide in normal blood and urine are described. Such determinations may be carried out on 1 ml. samples. After administration of bromide the determination can be carried out on finger blood (0.2 ml.). Bromide added to blood and urine can be quantitatively recovered.

6. The concentration of bromide in normal blood varied for 8 students between 227 and 572 γ /100 ml. The mean values for blood and urine were 372 and 656 γ /100 ml. respectively, the average chloride concentration of the urine being 605 mg./100 ml. The average Br/Cl ratio in urine was 1:2150, the halogen concentrations being expressed as milli-equiv.

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CIII. METABOLISM OF POLYCYCLIC COMPOUNDS.

II. PRODUCTION OF DIHYDROXYDIHYDROANTHRACENEGLYCURONIC ACID FROM ANTHRACENE.

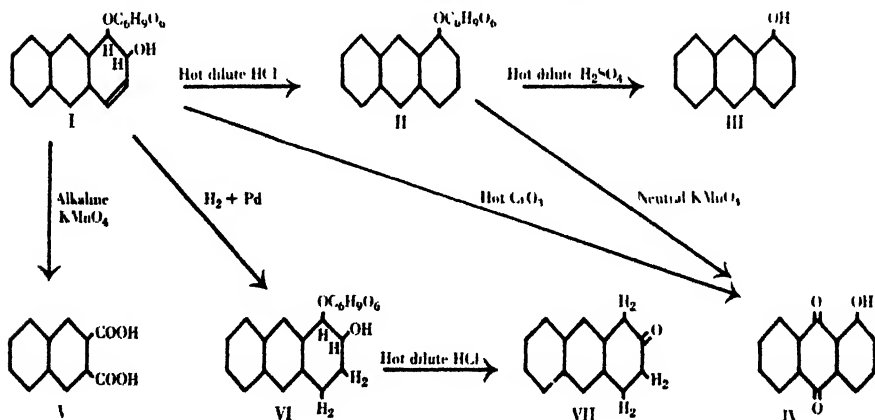
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In a previous communication it was shown that rats and rabbits fed on a diet containing anthracene excreted two different isomerides of dihydroxydihydroanthracene [Boyland and Levi, 1935]. These compounds were excreted as such and as conjugation compounds with glycuronic acid. The structure of the glycuronic acid compounds has now been determined.

The glycuronic acid compound from rabbit urine was oxidised by alkaline KMnO_4 to naphthalene-2:3-dicarboxylic acid (V), but treatment with acid caused loss of water to give an anthrylglycuronic acid (II), which was oxidised



by KMnO_4 to give α -hydroxyanthraquinone (IV). The acid must therefore be 1:2-dihydroxy-1:2-dihydroanthracene-1-glycuronic acid (I). That the oxygen atoms are attached in the 1 and 2 positions is shown by the formation of α -anthrol (III) by acid hydrolysis, and 2-keto-1:2:3:4-tetrahydroanthracene (VII) by catalytic reduction and acid hydrolysis.

The glycuronic acid from the rat urine occurred in much smaller amounts and was isolated with considerable difficulty from the acid solution. The purified product showed the chemical properties of α -anthrylglycuronic acid, but its optical activity was somewhat greater than that of the α -anthrylglycuronic acid (II) produced by acid hydrolysis of 1:2-dihydroxy-1:2-dihydroanthraceneglycuronic acid obtained from rabbit urine. It is probable that the rat urine contains a laevorotatory 1:2-dihydroxy-1:2-dihydroanthraceneglycuronic acid.

Of the two 1:2-dihydroxy-1:2-dihydroanthracenes the laevorotatory compound is excreted by rats, and it is probable that a glycuronic acid complex of this is formed. Rats excrete chiefly (0.2 g./l.) free 1:2-dihydroxy-1:2-dihydroanthracene and very little glycuronic acid. Rabbits produce little free 1:2-dihydroxy-1:2-dihydroanthracene and considerable amounts (0.5 g./l.) of the glycuronic acid complex.

EXPERIMENTAL.

The animals were treated in the manner described in the previous communication. After the neutral urine had been extracted with ether to remove dihydroxy-dihydroanthracene it was acidified with HCl until acid to Congo red and extracted with CHCl_3 in large separating funnels to remove mercapturic and other acids. The urine was then shaken twice with 5 g. lots of charcoal (norite), and filtered.

Glycuronic acid from rabbit urine.

The rabbit urine contained relatively large amounts of glycuronic acid (about 0.5 g. l.), which was readily extracted by shaking with 2 portions of amyl alcohol in a separating funnel. The extraction was facilitated by the addition of about 300 g. $(\text{NH}_4)_2\text{SO}_4$ l. The amyl alcohol was then extracted with 2N NaOH. The extract on acidifying and stirring gave a precipitate of crude glycuronic acid which was purified by crystallisation from hot water or hot alcohol.

The purified extract from acidified rabbit urine crystallised from hot water in laminae, M.P. 197° with decomposition, and from alcohol in rosettes or needles, M.P. $193\text{--}195^\circ$; $[\alpha]_D^{20}$ in dioxan (c, 0.6%) $+197^\circ$; $[\alpha]_D^{20}$ Na salt in H_2O (c, 0.3%) $+95^\circ$. Soluble in acetone and alcohol, sparingly soluble in ether and water; insoluble in benzene and chloroform. (Found (Schoeller): C, 59.4; H, 5.1%; equiv. wt. 408, 412, 407. $(\text{C}_{20}\text{H}_{20}\text{O}_8, \text{H}_2\text{O})$ requires C, 59.2; H, 5.4%; mol. wt. 406.)

The pure substance gave a strong naphthoresorcinol test for glycuronic acid. It did not reduce Fehling's solution except after acid hydrolysis. Boiling dil. HCl for 5 min. changed the optical activity and gave a less soluble product which appeared to be anthrylglycuronic acid (II).

Anthrylglycuronic acid. For the preparation of this substance the glycuronic acid of the rabbit is dissolved in hot water and, after the addition of a little HCl, kept at about 70° for an hour. The anthrylglycuronic acid slowly separates in shining laminae and is removed from the cooled solution, washed with benzene to remove a small amount of α -anthrol and purified from a large volume of water, or from aqueous methyl alcohol; $[\alpha]_D^{20}$ in dioxan (c, 1.0%) -52° ; $[\alpha]_D^{20}$ Na salt in H_2O (c, 0.3%) -79° . Readily soluble in alcohol and acetone, sparingly in water and ether. The solubility is greater in alcohol and less in water than that of the original acid. (Found (Weiler): C, 62.6; H, 5.1%; equiv. wt. 392. $\text{C}_{20}\text{H}_{18}\text{O}_7, \text{H}_2\text{O}$ requires C, 61.9; H, 5.1%; mol. wt. 388.)

The anthrylglycuronic acid (0.5 g.) was hydrolysed by boiling with 0.2N H_2SO_4 for 4 hours. On cooling, the precipitate was filtered and extracted with benzene. The extract gave crude α -anthrol (III) (0.1 g.), which was acetylated with cold acetic anhydride and pyridine. The α -acetoxyanthracene was purified by sublimation *in vacuo* giving needles, M.P. 127° (α -acetoxyanthracene has M.P. $128\text{--}130^\circ$).

The residue after benzene extraction of the hydrolysis product was readily soluble in alcohol and was recrystallised from water. It appeared to be unchanged anthrylglycuronic acid; $[\alpha]_D^{20}$ in dioxan (c, 0.8%) -50° . This appears to be identical with the anthrylglycuronic acid obtained by short boiling of the

original acid with dilute mineral acid and indicates that this acid is much more resistant to acid hydrolysis than other glycuronic acids, such as bornylglycuronic acid [Quick, 1927].

Oxidation of the original glycuronic acid with CrO_3 in acetic acid at 100° gave a yellow crystalline product which was recrystallised from aqueous alcohol, and resublimed *in vacuo*, M.P. $185\text{--}186^\circ$ (α -dihydroxyanthraquinone has M.P. 193°). It was soluble in alkali with an orange colour and appeared to be α -hydroxyanthraquinone (IV). Oxidation of α -anthrylglycuronic acid (obtained by acid hydrolysis of the original acid) with KMnO_4 in alkaline solution gave the same α -hydroxyanthraquinone (IV). This was purified by sublimation *in vacuo* and formed orange needles, M.P. 193° .

Oxidation of the original glycuronic acid with alkaline KMnO_4 gave naphthalene-2:3-dicarboxylic acid (V), M.P. $236\text{--}238^\circ$ (M.P. in literature 240°); equiv. wt. 114 (theory = 108). On vacuum sublimation this gave the anhydride, M.P. $242\text{--}244^\circ$ (M.P. in literature 245°).

The glycuronic acid was not hydrogenated under the conditions which were effective with 1:2-dihydroxy-1:2-dihydroanthracene (*i.e.* using a Pd-BaSO_4 catalyst), but using dioxan as solvent and a Pd catalyst prepared by reduction of PdCl_2 with sodium formate the glycuronic acid took up H_2 . The hydrogenation product was much less soluble in dioxan and acetone than the original acid and crystallised from hot water, M.P. 188° ; $[\alpha]_D$ Na salt in water (*c.* 0.14 %) -41° . (Found (Weiler): C, 56.6; H, 6.2 %; equiv. wt. 420. $\text{C}_{20}\text{H}_{22}\text{O}_8$, $2\text{H}_2\text{O}$ requires C, 56.3; H, 6.1 %; mol. wt. 426.)

It appeared to have one molecule of hydrogen more than the original acid and was probably 1:2-dihydroxy-1:2:3:4-tetrahydroanthraceneglycuronic acid (VI).

The reduced glycuronic acid was rapidly changed with boiling 2*N* HCl and gave 2-keto-1:2:3:4-tetrahydroanthracene (VII), M.P. 149° [Brown and Bayer, 1926], identical as to M.P. with that obtained from 1:2-dihydroxy-1:2:3:4-tetrahydroanthracene [Boyland and Levi, 1935]. The keto-compound could not be recrystallised satisfactorily. (Found (Weiler): C, 85.0; H, 6.4 %; $\text{C}_{14}\text{H}_{12}\text{O}$ requires C, 86.2; H, 6.1 %.)

Glycuronic acid from rat urine.

The rat urine gave a much less intense glycuronic acid reaction with naphthoresorcinol than the rabbit urine. After removal of the 1:2-dihydroxy-1:2-dihydroanthracene by ether extraction, the urine was acidified with HCl and extracted with ether in continuous extractors for 2 or 3 days. Small amounts of crystalline glycuronic acid separated from the ether. The yields were much smaller than those obtained from rabbit urine. The glycuronic acid was repeatedly crystallised from hot water and was finally obtained in laminae, M.P. $199\text{--}200^\circ$ with decomposition; $[\alpha]_D$ in dioxan (*c.* 0.5 %) -114° ; $[\alpha]_D$ Na salt in H_2O (*c.* 0.3 %) -126° .

Soluble with violet fluorescence in alcohol and acetone but not in benzene. (Found (Schoeller): C, 61.3; H, 5.05 %; equiv. wt. 392. $\text{C}_{20}\text{H}_{20}\text{O}_8$ requires C, 61.8; H, 5.15 %; mol. wt. 388.)

The pure substance gave a strong naphthoresorcinol reaction for glycuronic acid. The glycuronic acid was heated in a sealed tube at 100° with 0.2*N* HCl for 24 hours. A dark green precipitate was filtered off, dried, dissolved in benzene and acetylated with cold acetic anhydride and pyridine. The acetoxyanthracene was sublimed *in vacuo* to give white crystals, M.P. 126° (α -acetoxyanthracene, M.P. $128\text{--}130^\circ$; β -acetoxyanthracene, M.P. 198°).

The glycuronic acid was oxidised with KMnO_4 in aqueous dioxan solution. The solution was decolorised with SO_2 and concentrated by evaporation. An orange precipitate was filtered off, dried and sublimed *in vacuo*. Orange needles, m.p. 193° , agreeing with that of α -hydroxyanthraquinone, m.p. 193° .

SUMMARY.

Rabbits fed on a diet containing anthracene excrete a dextrorotatory 1:2-dihydroxy-1:2-dihydroanthracene-1-glycuronic acid. Rats fed on the same diet excrete much less glycuronic acid, but it is probable that they excrete an analogous laevorotatory compound which is readily hydrolysed to α -anthryl-glycuronic acid.

One of us, A.A.L., has pleasure in thanking the Sir Halley Stewart Trust for a Fellowship held during the progress of this work.

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CIV. MICRO-DETERMINATION OF URIC ACID.

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PRINCIPLE OF THE METHOD.

IN studying uric acid synthesis in avian tissues a reliable micro-method for the determination of uric acid is desirable. Folin's colorimetric method and its modifications which are fairly reliable for blood, and possibly for normal urine, are very unsatisfactory in presence of tissue extracts. The colour-producing reaction lacks specificity [Fujiwara and Kataoka, 1933] and the colour developed is not wholly proportional to uric acid concentration. A second method developed by Fosse *et al.* [1930] depends on the ultimate quantitative conversion of uric acid into urea and glyoxylic acid by the enzymes uricase and allantoinase. With the enzyme preparations at our disposal, however, the analysis takes too long (12 hours). We first tried to improve this enzymic method but finally abandoned it in favour of a similar but non-enzymic procedure.

In the method described in this paper uric acid is decomposed by the following reactions:

- | | | |
|----|-----------------------|---|
| 1. | Uric acid
↓ | shaken in air with MnO_2 ($p_{\text{H}} \sim 13$; 40) |
| 2. | Allantoin
↓ | alkaline hydrolysis (100° ; $p_{\text{H}} \sim 10$; 20 min.) |
| 3. | Allantoic acid
↓ | acid hydrolysis (100° ; $p_{\text{H}} \sim 2$; 3 min.) |
| | Urea + glyoxylic acid | |

Either end-product can be determined with ease, glyoxylic acid colorimetrically [Ro, 1932; Mourot, 1935] or by bisulphite-titration, urea by many methods. We prefer to determine urea manometrically by means of urease, since this method is specific, simple and accurate. Though the yield of urea is not theoretical, the deficit is constant ($8 \pm 3\%$) and can be allowed for by a correction factor. In practice $237 \mu\text{l. CO}_2$ per mg. uric acid are obtained instead of $265 \mu\text{l. CO}_2$. Since the error in determining CO_2 manometrically is $1\text{--}2 \mu\text{l.}$, the method allows $0.1\text{--}1.0$ mg. uric acid to be determined with reasonable accuracy (Table III).

The method is particularly suitable for use in conjunction with tissue slice work [Edson *et al.* 1935]. It is not suitable for blood analysis, nor is it recommended for very dilute solutions containing less than 0.1 mg. uric acid in 5 ml. of fluid.

EXPERIMENTAL.

I. Reagents.

1. *Manganese dioxide.* 10 ml. 20 vols. % H_2O_2 are added to 10 ml. of 1.82% KMnO_4 solution. When effervescence has subsided, the precipitate is separated by centrifuging, washed first with water and then with 0.1 M NaOH and finally suspended in 100 ml. 0.1 M NaOH . When the precipitate is uniformly distributed 0.1 ml. of this suspension contains 0.1 mg. MnO_2 . The suspension retains its activity for several weeks; it has to be uniformly distributed before use.

2. *2 N sodium hydroxide.*

3. *N sodium bicarbonate.*

4. *Thymol blue* (0.2% in 70% alcohol).
5. 6 N *sulphuric acid*.
6. 3 N *sodium acetate*.
7. 3 M *acetate buffer*, 27.2 g. sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) and 6.0 g. glacial acetic acid in 100 ml. p_{H} 5.0.
8. *Naphthyl red* (0.25 % in 70 % alcohol).
9. *Urease*: 1 part of jack bean meal (Arlico or British Drug Houses, Ltd.) is shaken for 30 min. with 4 parts of water. After centrifuging, 0.1 volume of acetate buffer is added to the separated supernatant fluid, which contains the enzyme. The solution retains its activity for several months if kept cool and if a drop of octyl alcohol is added to maintain sterility.

II. Details of the reactions involved.

Oxidation of uric acid. Schuler and Reindel [1933] have shown that uric acid is oxidised catalytically by molecular oxygen in the presence of MnO_2 ; the reaction depends on temperature, p_{H} of the medium and the concentration and method of preparation of the catalyst. The dried MnO_2 of British Drug Houses, Ltd. is almost completely inactive under conditions in which freshly precipitated catalyst is highly active. 0.3 ml. of the suspension described in Section I oxidises, under the given conditions, 1–2 mg. uric acid in 30 min. (Table I).

Table I. *Catalytic oxidation of uric acid with MnO_2 .*

Manometric experiment; conical cup with side bulbs; main part of cup contained 2 ml. uric acid solution (= 1.895 mg. uric acid) + 0.1 ml. 2N NaOH; side bulbs, 0.3 ml. MnO_2 suspension: 40°; gas, air; MnO_2 and uric acid mixed after equilibration.

Time after mixing (min.)	5	10	20	40	80	
Oxygen absorbed ($\mu\text{l.}$)	33.4	65	123	126.5	126.5	(calculated 126.5)

Hydrolysis of allantoin. The conditions established by Ro [1932] for the alkaline and acid hydrolysis of allantoin have been found satisfactory for our purpose. Table II shows that the method yields 96–98% of the theoretical amount of urea. Allan and Cerecedo [1931] showed that the yield of urea can be increased to 100% if the hydrolysis is carried out at 70°. Allan and Cerecedo's procedure, however, requires 2.5 hours.

Table II. *Hydrolysis of allantoin (20 min. p_{H} 10; 3 min. p_{H} 2).*

Allantoin mg.	Urea found (expressed in $\mu\text{l. CO}_2$)	Yield %
0.284	77.7	97.0
0.448	123.0	97.3
0.493	135.0	97.2
0.697	197.0	96.6

III. Procedure.

3–5 ml. of solution containing uric acid are transferred to a conical Warburg cup. If these cups are being used in a tissue slice experiment, the slices are simply removed from the solution. Sufficient 2N NaOH is added to the solution to make the fluid approximately 0.1 N with respect to OH^- ; 0.3 ml. MnO_2 suspension is then added, and the vessel is attached to the manometer and shaken for 40 min. at 37–40°. The gas space contains air. The MnO_2 is then separated from the solution by filtration, the filtrate is collected in a 10 ml. graduated test-tube and its volume read carefully. If the diameter of the filter-paper is 4 cm. and the filtrate is allowed to drain thoroughly, the loss of fluid is about 0.3–0.4 ml.

Next 0.1 volume of N NaHCO_3 solution is added to reduce alkalinity. The tube is heated for 20 min. in a boiling water-bath, a drop of thymol blue is added, and when cool the fluid is cautiously acidified by addition, drop by drop, of $6N$ H_2SO_4 until the indicator is distinctly red (p_{H} 2). The tube is returned to the boiling water-bath for 3 min. and cooled. A drop of naphthyl red is added and p_{H} is adjusted to 5.0 by $3M$ sodium acetate; a drop of both indicators added to acetate buffer of p_{H} 5.0 serves as a standard colour. An aliquot of the solution is then transferred to a conical Warburg flask and urea is determined with urease by the method of Krebs and Henseleit [1932].

IV. Example.

3 ml. of solution containing 1.450 mg. uric acid were shaken with 0.3 ml. MnO_2 suspension and 0.15 ml. $2N$ NaOH (40 min.: 40°).

Filtrate = 3.0 ml. (factor for correction $\frac{3.45}{3.00}$) + 0.3 ml. N NaHCO_3 .
 ↓
 Heated to 100° for 20 min.
 ↓ cooling, + 1 drop thymol blue + $6N$ H_2SO_4 until red.
 Heated to 100° for 3 min.
 ↓ cooling, + 1 drop naphthyl red + 40% sodium acetate until the colour of the control is reached.
 ↓ Total volume now 5.1 ml. Transferred into manometric flask for urea determination: 5.0 ml. (factor for correction $\frac{5.1}{5.0}$).
 ↓ Urea determination: 0.4 ml. urease; V_{P} 5.4 ml.; V_{O} 11.17; 38. K_{CO_2} 1.273.
 Pressure changes after mixing: time 20 40 60 min.
 mm. 213.5 238 238.5

$$V_{\text{CO}_2} = 238.5 \times 1.273 \times \frac{3.45}{3.00} \times \frac{5.1}{5.0} = 357 \mu\text{l.}$$

 (theoretical value 385; yield 92.7%).

V. Recovery.

In Table III a number of recovery experiments, carried out on pure uric acid solutions, are listed. The average yield is 92.1%, if the first two determinations, in which the amount of uric acid was small and the accuracy therefore less, are excluded. The last column of Table III shows that the deviations from the average figure are, with one exception, less than 3%. We correct this deficit in the uric acid determinations by dividing the CO_2 found by 0.92.

Table III. *Recovery of uric acid.*

Uric acid added mg.	CO_2 found $\mu\text{l.}$	Yield %
0.073	18.8	97
0.146	39.1	101
0.182	45.7	94.8
0.241	59.7	93.3
0.253	63	94.0
0.255	62.5	92.5
0.292	69.2	89.2
0.328	82.0	94.3
0.484	112	87.5
0.507	122	91.1
0.730	179	92.4
0.967	236	92.2
1.450	357	92.7
1.930	482	94.0
1.930	469	91.5
2.42	577	90.5
		Average 92.1%

VI. *Interference by other substances.*

The recovery of uric acid is equally satisfactory, if liver slices, liver extract or dilute (1 : 10) serum are present. Higher concentration of proteins, above 1–2 %, interfere with the filtrations. Agglomeration of MnO_2 , which may occur in certain protein-containing solutions, does not significantly affect the catalytic activity.

If the solution contains glucose, and possibly other substances, autoxidisable products are formed in the course of the alkaline hydrolysis. These cause an absorption of oxygen during the urea determination, but this difficulty can be overcome by filling the manometric flask with nitrogen.

If urea is present, it is advisable either to determine it directly in an aliquot or to destroy it by means of urease before the determination of uric acid.

VII. *Specificity.*

The following purines and pyrimidines do not yield urea when treated according to Section III and do not absorb oxygen with MnO_2 in alkaline solution: hypoxanthine, xanthine, adenine, guanine, cytosine, uracil, thymine.

Substances which may interfere with the determination of uric acid are a number of guanidine derivatives (arginine, glycoyamine, guanidine and creatinine) and some ureides (such as allantoin, allantoic acid, parabanic acid, oxaluric acid and dialuric acid). Table IV shows the extent to which urea may

Table IV. *Production of urea from substances other than uric acid.*

Substance	Urea formed when treated according to section III, %, maximum yield
Allantoin	98
Allantoic acid	98
Parabanic acid	98
Dialuric acid	98
Arginine	9
Guanidine	11
Creatinine	10
Glycoyamine	10
Oxaluric	98

be formed from these substances under the conditions of the determination. In all these cases the urea is produced during the alkaline or acid hydrolysis so that uric acid can be determined in their presence if the urea formed in an aliquot, without previous treatment with manganese dioxide, is measured.

SUMMARY.

A method is described, by which 0.1–2.0 mg. uric acid can be determined with reasonable accuracy. Uric acid is converted into urea and glyoxylic acid and the urea produced is estimated manometrically by means of urease. The method is especially suitable for use in conjunction with tissue slice work.

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CV. POTENTIOMETRIC TITRATIONS OF VITAMIN B₁ AND OF THIOCHROME.

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INTRODUCTION.

THIS work is a continuation of that of Moggridge and Ogston [1935]. It embodies titration curves of vitamin B₁ in more concentrated solution and of thiochrome [Kuhn and Vetter, 1935; Barger *et al.*, 1935], spectrographic measurements and catatorulin tests. Thiochrome is of interest since it is an oxidation product of the vitamin differing by only 2 or possibly 3 H atoms.

RESULTS AND DISCUSSION.

Vitamin B₁. The vitamin combines with only two equivalents of acid. The formula proposed by Williams [1935] demands that one of these shall be combined with a quaternary nitrogen of unusual type forming part of a thiazole group. The structure of the latter appears to have been completely settled by the synthesis of Clarke and Gurin [1935]. The peculiar N atom determines the character of a pseudo-acidic or pseudo-basic group which can be titrated in the neighbourhood of p_{H} 9.0 [Birch and Harris, 1935; Moggridge and Ogston,² 1935; Williams and Ruchle, 1935]. This group it is agreed requires two equivalents of alkali. All these workers agree that a further group is present which can be titrated near p_{H} 4.8, but Moggridge and Ogston described also a basic group of p_{K} 3.4. But this is inconsistent with the remainder of the evidence.

The present results show clearly that the titration curve of vitamin B₁ below p_{H} 7.0 is satisfactorily accounted for by a single group of p_{K} 4.8. The finding of Moggridge and Ogston was due therefore to the fact that too dilute solutions were employed; either there was some decomposition of the vitamin or possibly an error in the large water correction. Hence all workers now agree that vitamin B₁ has p_{K_1} 4.8 corresponding to a basic group and p_{K_2} about 9.0 corresponding to a pseudo-acidic or pseudo-basic group, the latter group requiring two equivalents.

The present results upon vitamin B₁ were obtained with the hydrogen electrode. They are consistent with the previous findings regarding p_{K} 4.8 obtained with the glass electrode; spectroscopic observations (Fig. 1, Curve 1) indicate that the vitamin structure is essentially intact after titration [cf. the curves of Holiday, 1935; Peters and Philpot, 1933]. On the other hand, exposure of a vitamin solution to the hydrogen electrode under the conditions of titration at p_{H} 7.0 for 12 hours in one experiment (5) reduced its catatorulin activity by 80 % without

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² These authors thought that this group must be regarded as pseudo-acidic because of the effect of alcohol upon the p_{K} . The group however is so unusual that the alcohol effect may well be anomalous.

causing any material change in the absorption spectrum. It is possible therefore that even in the shorter period of titration (30 min.) the vitamin may be slightly

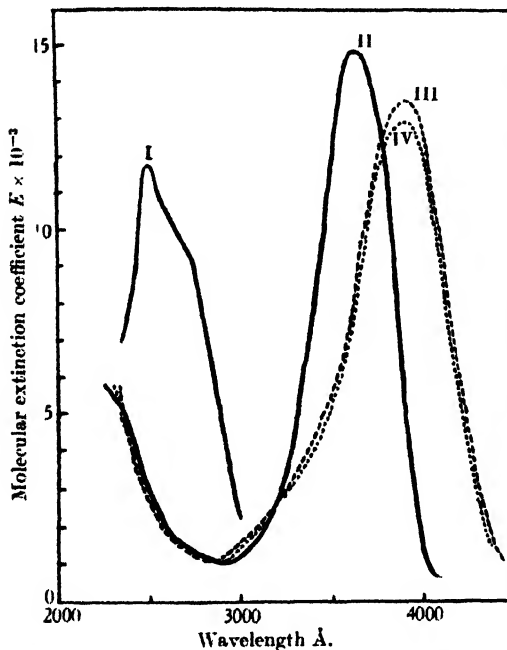


Fig. 1. Ultraviolet absorption spectra. Curve I. Vitamin at p_H 3 after having been in contact with H_2/Pt in the titration vessel. Curve II. Thiochrome at p_H 8. Curve III. Thiochrome at p_H 3. Curve IV. Thiochrome at p_H 3 after titration with the glass electrode.

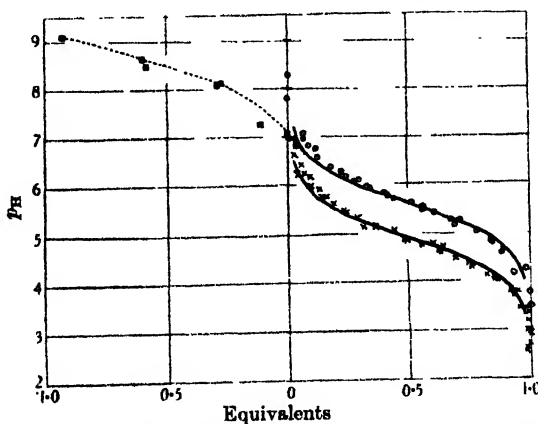


Fig. 2. Corrected titration curves of vitamin B₁ and thiochrome. \times Experimental points for vitamin B₁ below p_H 7. Full line is theoretical for 1 equiv. of p_K 4.8. \circ Experimental points for thiochrome; full line theoretical for 1 equiv. p_K 5.6. \square Equilibrium points for thiochrome.

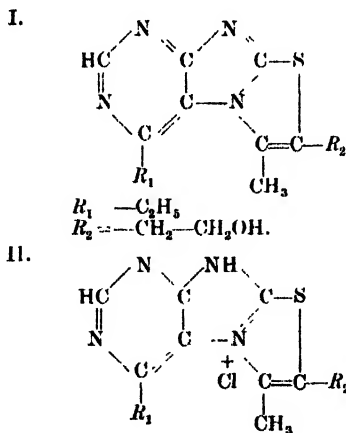
altered. It has always been found that the colour reaction is affected [cf. Moggridge and Ogston, 1935]. Kinnersley *et al.* [1935, 2] suggested that the group which can be titrated near p_H 4.8 contains quaternary nitrogen. The

elimination of the group of p_K 3.7 now means that we must return to the original view of Moggridge and Ogston that the group of p_K 4.8 is the amino-group attached to the pyrimidine ring. Since this group remains intact to the titration while the colour reaction disappears, it seems that the amino-group cannot be responsible for the diazo-reaction as has been suggested by Kinnersley *et al.* and Barger *et al.*

Thiochrome. Proper determination of the titration curve of thiochrome with the hydrogen electrode proved impossible, the curves being of indefinite form and changing with successive titrations of the same sample; this was associated with a change in absorption, diminution of the long-wave band and the appearance of a new band at about 2700 Å. This is a confirmation of the band given by Kuhn and Vetter [1935] for reduced thiochrome. Using a glass electrode however excellent titration curves (Fig. 2) were obtained, very similar to those of the vitamin; the ultra-violet absorption curve was not changed during titration (Fig. 1); there is a single group with p_K 5.6 the acid binding corresponding to one equivalent per mol. thiochrome (assumed to be $C_{12}H_{14}ON_4S=264$). Its titration seemed to correspond closely with the change from yellow to colourless of the solution. Between p_H 7.0 and 9.0 (the limit of the glass electrode) the solution behaved somewhat similarly to the vitamin; the p_H values drifted to equilibrium indicating the presence of a pseudo-acidic or pseudo-basic group titrating between p_H 8 and 10.

From determination of the fluorescence changes Kuhn and Vetter [1935] found a p_K of 6.3 for a basic group of thiochrome. Our data give a rather more acid value of 5.6.

Let us consider the interpretation of these results in relation to the recent formula proposed for thiochrome [Barger *et al.*, 1935; Kuhn and Vetter, 1935; Windaus *et al.*, 1934] with formation of a central glyoxaline ring (I). From the



titration data it seems that the p_K 5.6 of thiochrome belongs to the same N atom at 8 attached to the pyrimidine ring; the change from 4.8 to 5.6 might accompany the ring closure. The value of 5.6 is consistent with the p_K values of known glyoxaline basic groups [Levy, 1935]. It seems probable that the drift at alkaline reaction has the same cause both with vitamin and thiochrome. Formula I does not account satisfactorily for these points; we suggest formula II with a quaternary N atom. As Barger *et al.* point out there are objections to the assumption of a quaternary N atom in thiochrome; but without assuming this grouping we

cannot account for the alkali drift, since according to Clarke and Gurin the opening of the ring and alkali drift are associated with the quaternary attachment of the thiazole nucleus. Both Kinnersley *et al.* and Barger *et al.* consider that the acquisition of blue fluorescence (quinochrome and thiochrome) involves loss of sulphur lability.¹ No formula seems completely satisfactory. Thiochrome has been found to combine with 2 mols. of HCl, one of these being held less closely than that of vitamin B₁. This would suggest a weaker base for the second HCl whereas actually the titration data indicate a stronger base of p_K 5.6.

The changed absorption spectrum found after titration with the hydrogen electrode is sufficiently similar to that of vitamin B₁ to suggest the application of the catatorulin test, though Barger *et al.* state that hydrogenated thiochrome is not vitamin B₁. The short-wave maximum 2700 Å. is not very near that of B₁ in acid solution at 2470 Å. A few catatorulin tests have been made upon thiochrome itself and specimens after reduction by contact with hydrogen and platinum for 4 hours (see Fig. 3). The thiochrome both from Germany and Edinburgh showed

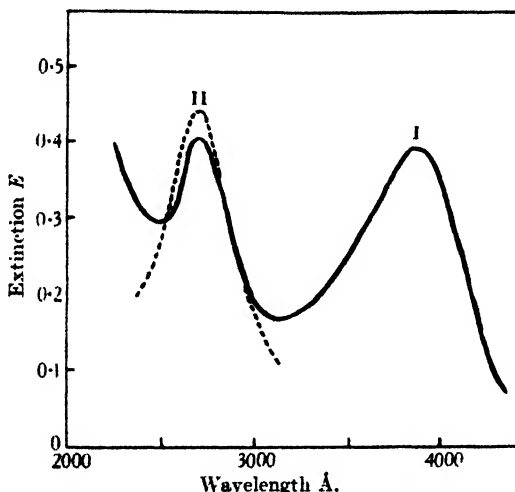


Fig. 3. Ultra-violet absorption spectra. Curve I. Thiochrome at p_H 3 after 4 hours' contact with H_2/Pt at p_H 7. Curve II. Thiochrome at p_H 3 after 4 hours' contact with H_2/Pt at p_H 5.5.

no catatorulin activity (Exps. 1 and 2) in confirmation of animal tests from these laboratories. Some specimens of reduced thiochrome (Exps. 3, 4, 5) showed a trace of apparent activity almost within limits of experimental error. This matter is being further investigated.

EXPERIMENTAL.

Hydrogen electrode titrations were performed in a small rocking half-cell (Fig. 4) working on the principles of that described by Clark [1920]. Its capacity was 0.15 ml. Hydrogen electrodes were small platinised plates [Ogston and Brown, 1935]. Hydrogen flowed over the surface of the liquid and escaped through a trap; contact of the liquid with hydrogen and the electrode and mixture of the liquid were assured by keeping the cell mechanically rocked through an

¹ On the other hand, Kuhn and Vetter state that after long boiling with 2N NaOH thiochrome liberates S as $-SH$.

angle of 20° about three times per sec. A 3.5 *N* calomel electrode was the reference electrode; the whole cell was in an air thermostat at $25 \pm 0.2^\circ$.

Glass electrodes were of the type described by MacInnes and Dole [1933] having very thin membranes of about 2 mm. diameter; they were calibrated against buffers standardised with the hydrogen electrode. Electrical measure-

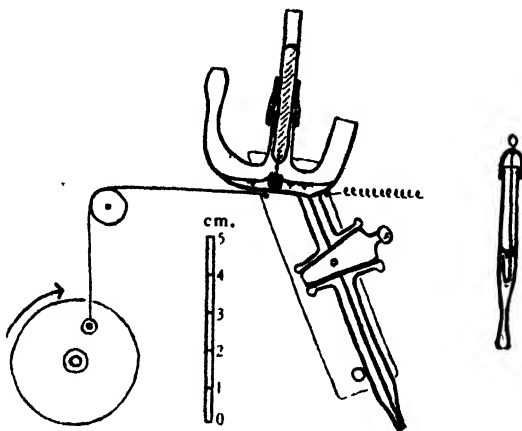


Fig. 4. Hydrogen electrode vessel.

ments were made with a Tinsley slide wire potentiometer using a Tinsley valve electrometer with glass electrodes. Potentials were measured to the nearest millivolt. Titrand solutions were approx. *M*/150; 0.1 *N* titrant solutions were added from a capillary microburette which allowed reading to $\pm 2 \times 10^{-5}$ ml.

Ultra-violet absorption spectra were obtained with a Hilger E 316 Quartz spectrograph with a Spekker photometer. All spectra were measured in solutions of about p_H 3 except that of thiochrome which was measured at p_H 8.

The amount of thiochrome used for these experiments was under 1.0 mg.

The vitamin was prepared in the Department of Biochemistry, Oxford University [Kinnersley *et al.*, 1935, 1]; the thiochrome was available to us by permission and through the courtesy of Prof. Kuhn and Prof. Barger. All observations upon thiochrome with the exception of the catatorulin tests, Exps. 1 and 2, were made with Prof. Kuhn's specimen.

Catatorulin tests. Medium: Ringer phosphate of p_H 7.3, no pyrophosphate; substrate: pyruvate instead of lactate. Figures represent average O_2 uptake $\mu\text{l./g./hr.}$ for a period of 1–2 hours.

		Additions	$\mu\text{l./g./hr.}$
Exp. 1		0	861
	$\mu\text{l. B}_1$	1 γ	1345
	Thiochrome Kuhn	5 γ	848
	Thiochrome Barger	5 γ	869
Exp. 2		0	556
	Vitamin B_1	1 γ	995
	Thiochrome Kuhn	5 γ	550
	Thiochrome Barger	5 γ	572

The results show that less than 1% vitamin B_1 activity (catatorulin) is present in the thiochrome preparations of Kuhn and Barger.

		Additions	$\mu\text{l./g./hr.}$	
Exp. 3		0	565	
	Vitamin B ₁	0.5 γ	973	
	Vitamin B ₁	1.0 γ	1016	
	Reduced thiochrome p_{H} 3.9 during titration	16 γ	679	Not more than 0.15 γ vitamin B ₁ present or 1%
Exp. 4		0	476	
	Vitamin B ₁	0.25 γ	675	
	Vitamin B ₁	0.5 γ	757	
	Reduced thiochrome p_{H} 7.5 (4 hours)	{ A 1/5	489	Nil
		{ B 1/5	508	Not more than 0.05 γ present
Exp. 5		0	618	
	Vitamin B ₁	0.25 γ	924	
		0.5 γ	1010	
		2 γ original B ₁ reduced p_{H} 7.0 (12 hours)	912	Not more than 0.25 γ or not more than 12%
		12 γ reduced thiochrome p_{H} 5.5 (4 hours)	772	0.12 γ or 1% present
Exp. 6		0	602	
	Vitamin B ₁	2 γ	1219	
		0 + 22 γ reduced thio- chrome p_{H} 2.5 (12 hours)	639	Not more than 0.05 γ or 1 in 500 probably
	Vitamin B ₁	2 + 22 γ thiochrome	1228	
	All reductions at 25° or room temperature with H ₂ and Pt black.			

SUMMARY.

1. The titration curve of vitamin B₁ on the acid side is completely described by one basic group having p_{K} 4.8. All workers are now in agreement.
2. Thiochrome (Kuhn) shows a basic p_{K} of 5.6 and like vitamin B₁ a tendency for a drifting titration value to the alkaline side of the neutral point. It has no vitamin B₁ activity by catatorulin test.

We are indebted to Mr H. W. Kinnersley for help.

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OBITUARY NOTICE.

GEORGE ALECK CROCKER GOUGH.

1902-1935.

GEORGE ALECK CROCKER GOUGH died on November 8th, 1935, after a prolonged illness.

Gough received his education at Battersea Secondary School from 1917 to 1919 and then proceeded to Battersea Polytechnic where he remained until 1926. In 1922 he obtained the B.Sc. degree with first class honours in Chemistry and three years later he was awarded a Ph.D. degree for a thesis embodying work that he had carried out in collaboration with Dr J. Kenyon and Dr H. Hunter on the Geometrical and Optical Isomerism of the Methylcyclohexanols (*J. Chem. Soc.* (1926), 2052).

Towards the end of 1926 Gough went to work in the Chemical Laboratory of the National Institute for Medical Research at Hampstead and here his talents were quickly recognised. In collaboration with Dr H. King he entered a new and difficult field of research and investigated the relation between the structure, the physical and chemical properties and the therapeutic activity in experimental trypanosome infection of some new organic compounds containing arsenic (*J. Chem. Soc.* (1928), 2426 and (1930), 669). During the preparation of nicotinic acid, with a view to the synthesis from it of certain arsenical compounds, an unknown by-product was obtained and was subsequently identified as 4-nitro-5 (3-pyridyl)-pyrazole. This compound was found to have originated as the result of a new type of nuclear conversion (*J. Chem. Soc.* (1931), 2968; (1933), 350).

In 1929 Gough was awarded a Rockefeller Travelling Fellowship. During its tenure he worked under Prof. Wieland at Munich, studying some of the less well-known sterols of yeast (*Liebigs Ann.* (1930), 482, 36). As soon as he had completed his studies Gough took a course of quantitative organic analysis under Prof. Fritz Pregl at the Medizinisch-Chemischen Institut of the University of Graz. He remained keenly interested in micro-chemical methods and after he had returned to England he gave several courses on this subject at the Sir John Cass Technical Institute in London.

When Gough resumed work at the National Institute he extended an investigation which commenced many years before, when the late Dr H. W. Dudley demonstrated the existence of a specific carbohydrate-hapten in tubercle bacilli. Gough devised an improved technique for the isolation of this serologically reactive substance from large quantities of organisms and obtained by this means sufficient material for a thorough chemical analysis. He showed that by mild acid hydrolysis it was possible to obtain from the polysaccharide material a mixture of sugars, of which arabinose, mannose and galactose were isolated and identified (*Biochem. J.* (1932), 26, 248). Gough also examined the water-soluble protein substances which had been derived from tubercle bacilli, and he succeeded in isolating them in an undenatured condition and showed that they possessed different chemical and immunological properties (*Biochem. J.* (1933), 27, 1048). Gough's work at this time also included an examination of the polysaccharide material of Timothy grass (*Phleum pratense*). The relationship of certain constituents of pollens to the allergic diseases to which they give rise led

Gough to examine the polysaccharide fraction that he had isolated from the pollen of Timothy grass since it seemed probable that this material was responsible for the specificity of the allergic reaction. He found that the polysaccharide displayed a certain similarity to the specific carbohydrate of the tubercle bacillus and that acid hydrolysis of the carbohydrate material produced *l*-arabinose, galactose and a non-reducing acid.

Gough next investigated the properties of two proteins that are found in the synthetic medium in which tubercle bacilli have been cultivated and he attempted to ascertain the part played by each in the "tuberculin" reaction. He showed that the reaction produced by old tuberculin when injected into the skin is associated with both fractions, one of which is a typical protein whereas the other is a proteose-like substance which is not precipitated by trichloroacetic acid (*Brit. J. Exp. Path.* (1934), **15**, 237).

Gough's last contribution to immuno-chemistry was concerned with the chemical nature of the phage-inactivating agent in bacterial extracts. In collaboration with Dr F. M. Burnet he showed that the active agent is a polysaccharide which can be converted into the specific bacterial hapten by treatment with alkali. The results of this work indicated that during the course of degradation of the phage-inactivating agent by heat and alkali the power to inactivate different phages is lost in definite stages. The evidence supported the belief that the specific somatic antigen of dysentery and of salmonella bacilli is a complex labile polysaccharide which is responsible both for the serological character and the phage-susceptibility of the bacteria (*J. Path. Bact.* (1934), **38**, 301).

Gough became an Associate of the Institute of Chemistry in 1924, a Fellow of the Chemical Society in 1926, and a Member of the Biochemical Society in 1931.

Gough was of a quiet and retiring disposition and was an enthusiastic lover of nature and of the open country. He took a keen and especial delight in his garden and in the beautiful countryside near his home in Surrey. His untimely death cuts short a promising future and removes an able and exceedingly conscientious worker from the field of immuno-chemistry. His loss will be deeply felt by all his friends and especially by the colleagues whom he had served so well.

W. T. J. M.

CVI. A TEST FOR THYMINE, WITH OBSERVATIONS ON THE KETO-ENOLIC TYPE OF DIAZO-TEST.

By GEORGE HUNTER.

From the Department of Biochemistry, University of Alberta, Canada.

(Received February 5th, 1936.)

FOR quantitative colorimetric purposes there is probably no other reagent so sensitive and so well adapted for precise measurements, and at the same time applicable in so many fields as diazotised sulphanilic acid. The present paper contains some observations, now some years old, on certain substances in many respects often widely different but which under similar conditions yield colours with this reagent. The group has the common character that all its members are capable of keto-enolic tautomerism for which reason I venture to speak of a keto-enolic type of diazo-test.

The reagents required for the tests are:

1. The diazo-reagent made according to Koessler and Hanke [1919].
2. 1.1 % sodium carbonate solution.
3. 3.0 *N* sodium hydroxide solution.
4. 20 % hydroxylamine hydrochloride in water.

Pyrimidines and diazo-tests.

The behaviour of pyrimidines with diazo-compounds has been most closely studied by Johnson and Clapp [1908-9]. Uracil, cytosine and especially thymine were found to give colours when mixed with diazobenzenesulphonic acid in presence of excess of sodium hydroxide. No reaction occurred with 3-methyl-pyrimidines, whence it was suggested that the grouping —CO—NH— must be present in the 2:1- or 2:3-positions of the pyrimidine ring to allow coupling. That coupling does not occur in the 4- or 5-positions was shown by the fact that 4:5-dimethyluracil gives a red colour.

Johnson and Clapp tried without success to isolate the reaction product of thymine and diazobenzenesulphonic acid. The nature of the linkage thus remains obscure although the available evidence favours the view that coupling takes place in position 3, in which case the reaction products would be *N*-azo-compounds. Johnson and Clapp further state that 6-hydroxy- and 6-amino-pyrimidine do not couple; Evans [1893] obtained a red colour with 2-oxy-4:6-dimethylpyrimidine, and Pauly [1904] an intense colour with 4-methyluracil. Steudel [1904] first observed that thymine couples with diazo-compounds in presence of sodium hydroxide.

The writer has confirmed the results of Johnson and Clapp with uracil, cytosine and thymine. It should be noted however that the colours are obtained only with relatively large amounts of the pyrimidines in question. The sensitivity of the tests can be raised slightly by the application of heat to the solutions, but it is unnecessary to emphasise this in view of the much more delicate test for thymine which will now be described.

The new test for thymine.

The test is performed as follows.

To 2.5 ml. of sodium carbonate solution add 1 ml. of the diazo-reagent. At the end of 1 min. add 0.5 ml. of a solution containing about 0.1 mg. thymine. Keep for 5 min., after which period a faint yellow colour is present. Add 1 ml. of 3 *N* NaOH and keep for 1 min. Then add 1 drop of the hydroxylamine solution and mix quickly. An intense red colour rapidly develops. It is stable for several hours.

The high sensitivity of the test is shown by the fact that a marked colour is still obtainable with less than 0.01 mg. thymine present in the test solution.

The test is not given by as much as 0.5 mg. uracil or cytosine, although yellow colours are produced by these in contact with the reagent and sodium carbonate.

It is necessary that coupling take place in sodium carbonate solution, since no colour is obtained at the above dilutions with excess of sodium hydroxide even on the addition of hydroxylamine.

It is difficult to assess the relevance of the present test to previous observations on the behaviour of pyrimidines with diazo-reagents. With Johnson and Clapp's procedure uracil and cytosine give positive tests though less intense than thymine, whilst the test now described quite definitely distinguishes thymine from uracil or cytosine. In the present test uracil and cytosine may couple, but if they do, the product is incapable of undergoing reduction to an intensely coloured complex as in the case of the thymine-coupled product.

The keto-enolic diazo-test.

Hanke and Koessler [1922] showed that if (in place of thymine in the test described above) 0.25 mg. acetone, acetaldehyde or acetoacetic acid were added, a purplish colour developed after the addition of hydroxylamine. The coupling was supposed to take place with the keto-form of these substances, the hydroxylamine playing an essential part in the production of the final red colour which was regarded as a hydrazoxime derivative.

The principle of the Hanke and Koessler test derives from the work of Ehrlich [1882], Penzoldt [1883, 1, 2] and Petri [1883] who independently found that under certain conditions the urine of diabetic patients gave a marked diazo-reaction which was traced to the presence of glucose.

According to Penzoldt 2 ml. of glucose solution (containing more than 0.1 %) were made strongly alkaline with KOH, and 2 ml. of 1.67 % diazobenzene-sulphonic acid, previously made slightly alkaline, added. In presence of a large amount of sugar a purplish colour developed after 15 min., but with small amounts of glucose only after an hour. A slight excess of nitrous acid did not interfere.

Penzoldt and Fischer [1883] showed that the test is a general one for aldehydes. Easily oxidisable aldehydes behaved like glucose, but aromatic aldehydes reacted only in presence of sodium amalgam, by the use of which a much greater colour was also obtained from glucose and acetaldehyde.

Positive tests were obtained by Penzoldt and Fischer with acetaldehyde, acetone, ethyl acetoacetate, valeraldehyde, furfuraldehyde, glyoxal and benzaldehyde. The same test was also given by phenol, resorcinol and pyrocatechol, if care were taken to bring them in contact with the diazo-compound in presence of excess of alkali so that the known dyes are not formed.

Petri [1883; 1883-84; 1884] added to 5 ml. of urine 2 ml. of 30 % KOH and 5 ml. of a diazo-reagent, made with approximately the theoretical amount of

potassium nitrite. He found that colour development was hastened by warming and was given only with fixed alkalis. Reduction with zinc in the absence of oxygen could replace reduction with sodium amalgam but over-reduction with zinc produced a colourless solution, which on exposure to oxygen rapidly became coloured again. Petri later found that peptones and proteins by the use of zinc give the same fuchsin-red colour as is obtained from aldehydes.

It appears probable that the glucose test is not given by glucose as such, but by substances of the type of acetaldehyde produced by relatively long contact with the strong alkali, although it is possible that enolisation [Nef, 1910] may cause the reaction to be given by glucose itself.

It thus appears that the tests described by Penzoldt and Fischer and by Petri for aldehydes are fundamentally the same as that described by Hanke and Koessler for acetone *etc.*, and that the hydroxylamine of the last workers replaces sodium amalgam or zinc as the reducing agent which is necessary for all the tests, except when, as with acetaldehyde, the substance itself has strong reducing properties.

The writer has confirmed the main findings of Penzoldt and of Petri, as well as those of Hanke and Koessler. It appears that the best conditions for the test with such a substance as acetone lie between those adopted by Penzoldt and by Hanke and Koessler. It is true that coupling does take place, especially with the aid of heat, in the presence of a large excess of strong alkali; but it is at best very slow and incomplete presumably owing to the fact that there is little active coupling diazo-compound present under such conditions. On the other hand in a dilute sodium carbonate solution of acetone there is probably very little of the enolic tautomeride, which according to Dimroth and Hartmann [1908] is the only form capable of coupling. The greater colour produced by acetone on prolonging the time of coupling in presence of dilute sodium carbonate, as found by Hanke and Koessler, or by the use of 10 % sodium carbonate instead of 1.1 % and keeping the coupling time 5 min. as found by the writer, is readily explicable on this hypothesis. A high concentration of weak alkali thus provides the best conditions for coupling as it does not greatly diminish the active diazo-compound and at the same time tends to increase the proportion of enolic form present.

The great variations in the extent of coupling of different substances of this type under such specific conditions as those adopted by Hanke and Koessler is also in accordance with the above explanation, the proportion of enolic form present under definite conditions being very variable for different substances.

It has further been found that many reducing agents may replace hydroxylamine in the production of the final colour. The addition, instead of hydroxylamine, of zinc, aluminium, tin or a drop of very dilute stannous chloride is quickly followed by the appearance of the red colour. In the case of stannous chloride the colour intensification with acetaldehyde is practically as great as that obtained with hydroxylamine. With the former, however, the colour is unstable. On keeping the test-tube, the colour fades rather rapidly except on the top layer. By shaking the contents it quickly returns, apparently to its full extent. The process may be repeated many times.

It is thus apparent that, although hydroxylamine may have more than a purely reducing function in the Hanke and Koessler test, yet it is not a necessary constituent in the general type of reaction under consideration. The Hanke and Koessler formulation is thus inadequate as an explanation of the mechanism of the reactions concerned.

It is probable that these proceed on the lines indicated by Dimroth and Hartmann [1908] for the coupling of diazonium salts with tribenzoylmethane.

According to this view the enolic compound would first form a yellow *O*-azo-derivative; migration of the arylazo-group to the neighbouring carbon atom would convert the latter compound into a deeply coloured *C*-azo-derivative which is unstable and passes spontaneously into the isomeric hydrazone (colourless).

The above series of reactions would represent the complete course of events in the absence of a reducing agent. The effect of such an agent may be explained by supposing that it converts the hydrazone into a hydrazide which is oxidised in turn by atmospheric oxygen with regeneration of the coloured *C*-azo-compound.

In the absence of direct proof of the existence of the above-mentioned intermediate products this explanation must be regarded as tentative; it appears however adequately to cover the observed facts.

Tyrosine and tyramine.

Hanke and Koessler [1922] discovered that tyrosine and tyramine give in a very beautiful fashion, and indeed quantitatively, the type of diazo-reaction under discussion. *p*-Hydroxyphenylpropionic and *p*-hydroxyphenyllactic acids behave as normal phenols, the presence of NH_2 in the side-chain giving rise to this remarkable difference in behaviour.

The writer has tested *p*-nitrophenol and *p*-aminophenol but with negative results. He has also found that tyrosine gives the Penzoldt aldehyde test but only in the presence of a metallic reducing agent. The use of stannous chloride in place of hydroxylamine hydrochloride in the Hauke and Koessler method gives the characteristic intensification of colour. As with the keto-enolic substances, the colour fades rather rapidly except on the surface layer and is quickly regenerated by shaking. As before the process can be repeated many times.

The parallel behaviour of tyrosine with the keto-enolic tautomerides thus leaves little doubt that the red compound produced in both cases is of the same type, and in the case of tyrosine is explicable by a set of reactions similar to that outlined for acetone. The initial formation of an *O*-azo-compound is in conformity with the conceptions of Chattaway and Hill [1922] of the mechanism of the coupling of phenols, but in the case of tyrosine, through the influence of the NH_2 -group in the side-chain, the migration of the arylazo-group is in some way effected, or the hydrazone structure is the more stable form as in the mixed azo-compounds. No azo-derivative of tyrosine has yet been described.

Thymine.

The test for thymine has already been described. Here again, the final colour behaves with stannous chloride as does that from acetone or tyrosine, and the colour produced by hydroxylamine is very stable.

The fact that uracil and cytosine do not give the test is perhaps no more remarkable than that *p*-hydroxyphenylpropionic acid and similar substances without an amino-group in the side-chain do not behave like tyrosine. The methyl group in the 5-position of thymine has an effect on the reaction apparently analogous to that of the amino-group in the side-chain of tyrosine. In the case of thymine, we must also suppose that the labile hydrogen migrates between a carbon and a nitrogen atom.

Vitamin B₁.

It is mentioned by Kinnersley *et al.* [1935] that their vitamin B₁ gives the keto-enolic type of diazo-test. As noted by these workers their test is given in a weak manner by acetone. It is more marked with acetaldehyde and glyoxal but

negative tests are obtained with tyrosine, thymine and acetoacetic acid. By their particular technique acetaldehyde gives a colour in presence of formaldehyde but none when the latter is replaced by hydroxylamine. The colour obtained from acetaldehyde by the so-called formaldehyde-azo test is less than that by the procedure described here. It is not stated by the Oxford workers if the colours obtained from vitamin B₁ by the two techniques are comparable in intensity.

SUMMARY.

1. A sensitive diazo-test for thymine has been described by which this pyrimidine may be distinguished from cytosine or uracil.

2. The term "keto-enolic type" of diazo-test is suggested to embrace a diversity of diazo-tests previously described as given by such substances as glucose, acetoacetic acid, acetaldehyde, acetone, tyrosine and now thymine, on evidence submitted that the tests all rest fundamentally on the same chemical principles.

3. The essentials for such a test appear to be (a) the capacity for keto-enolic tautomerism in the substance to be coupled; (b) the necessity for a reducing agent in the development of significant colour from the product of coupling.

I am indebted to Prof. Treat B. Johnson of Yale for the synthetic thymine used.

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CVII. THE PRECIPITATION OF CYSTINE BY PHOSPHOTUNGSTIC ACID.

By MANAYATH DAMODARAN
AND TARAKAD GANAPATHY SIVASWAMY.

From the University Biochemical Laboratory, Chempauk, Madras.

(Received February 10th, 1936.)

ATTENTION was drawn in the previous communication [Damodaran and Sivaswamy, 1936] to the fact that in analysing the globulin anacardein according to the Van Slyke nitrogen distribution method the precipitate obtained with phosphotungstic acid was completely free from sulphur, though the presence of cystine in the protein was established both qualitatively and quantitatively. The existing literature on the subject (for a historical summary see Hoffman and Gortner [1922]) would give the impression that the precipitation of cystine by phosphotungstic acid is nearly quantitative except when the former has been subjected to prolonged boiling with acid. Thus Van Slyke [1911] records a recovery of 88.8%. Hoffman and Gortner [1922] of 89.7%, and Plimmer and Lowndes [1927] of 97% of cystine in the phosphotungstic acid precipitate. According to the first two authors, the precipitability of cystine is reduced by boiling with concentrated hydrochloric acid for 24 hours to 50 and 56.78% respectively of the cystine originally present. Plimmer found that after similar treatment for 36 hours the phosphotungstic acid precipitate represented 40% of the cystine-S. These observations are however incapable of explaining the complete absence of sulphur from the phosphotungstic acid precipitate observed in the case cited above. Such an observation could only be understood if the precipitability of cystine were influenced by its concentration and there existed a certain minimum value of the latter below which cystine failed to give a precipitate with phosphotungstic acid. No systematic investigation seems to have been carried out from this point of view and it was considered advisable to study this question, especially as cystine-N values based on the sulphur content of the phosphotungstic acid precipitate obtained in the Van Slyke method continue to be widely quoted and the point is of importance in view of the occurrence of more than one sulphur-containing amino-acid in proteins.

Solutions of pure cystine in varying concentrations were treated with phosphotungstic acid under exactly the same conditions as in the usual nitrogen distribution method and the amount of precipitation taking place was determined. Experiments were carried out at three different temperatures, viz. at room temperature, which during the period varied from 29 to 30°, in a thermostat at 20° and at the temperature of the refrigerator (5–8°).

The results are represented graphically in Fig. 1.

It is obvious that precipitability is considerably influenced both by the temperature and by the concentration of cystine, and that within certain ranges of the latter no precipitation takes place. From the shape of the curves it can be inferred that at no concentration is the precipitation likely to be complete. By extrapolation it is possible to estimate the limiting concentrations at the various temperatures below which no precipitation would take place. These are approximately at 5–8° 3.1 mg., at 20° 16.9 mg. and at 29–30° 50 mg. of cystine per 100 ml. of solution. Translated into terms of protein, assuming the standard conditions

of the Van Slyke method, where 3 g. of protein are hydrolysed and the "bases" precipitated in a volume of 200 ml. at about 20° the results obtained show that a protein containing less than 1.13 % by weight of cystine would give no precipitate of cystine phosphotungstate, whilst from one containing as much as 5 % of this amino-acid only about 81.4 % would be precipitated. In the experiments of the authors already mentioned only one concentration of cystine was tried in

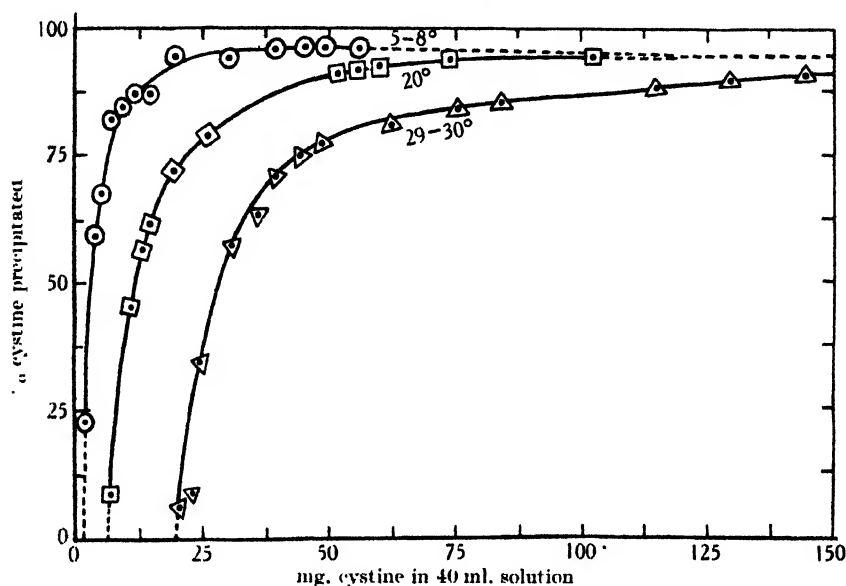


Fig. 1. Precipitation curves of cystine at different temperatures.

each case. Van Slyke [1911] as well as Hoffman and Gortner [1922] (with whose results the values now obtained are in close agreement at the particular concentration tried by them) used a solution containing 0.1 g. cystine per 100 ml.; the experiments of Plimmer and Lowndes [1927] were carried out on a solution containing 0.25 g. in 200 ml. These figures correspond to 3 g. of protein with cystine contents of 6.66 % and 8.33 % respectively. Since no protein, apart from some keratins, contains such large proportions of cystine, the results obtained by the above authors at the high concentrations investigated by them have no bearing on the applicability of the process to the analysis of protein hydrolysates. It also follows that the solubility correction of 0.0026 g. to be added to the cystine-N, recommended by Van Slyke on the basis of his experiments on the cystine solution of the strength mentioned above, is purely arbitrary and of little significance. Further the extent of the alteration or decomposition of cystine during treatment with acid as determined by Van Slyke and by Plimmer and Lowndes is subject to correction, inasmuch as these authors assumed that precipitability by phosphotungstic acid was a measure of unaltered cystine.

It will be seen that at the temperature of the refrigerator almost quantitative precipitation can be obtained. This is in conformity with the results recently published by Toennies and Elliott [1935] who carried out the precipitation at 0°. But advantage cannot be taken of this fact in the analysis of proteins, as at such low temperatures the nitrogen content of the phosphotungstic acid precipitate is out of all proportion to the basic amino-acids present.

EXPERIMENTAL.

Cystine was prepared from human hair without the use of sodium acetate for precipitation. After two recrystallisations, using sodium hydroxide for neutralisation, the preparation was analysed. (Found: N, 11.61; S, 26.58%. $C_6H_{12}O_4N_2S_2$ requires: N, 11.66; S, 26.67%.) For precipitation, solutions of the cystine were made up in varying concentrations in $N/5$ hydrochloric acid and the exact quantity of the amino-acid present ascertained by micro-Kjeldahl determinations on aliquots. Known volumes of the solution were pipetted out into stoppered pyrex bottles calibrated to contain 40 ml., 20 ml. of a solution containing 3 g. of 24-phosphotungstic acid (Kahlbaum) and 3.6 g. concentrated hydrochloric acid were added and the volume was made up with water to exactly 40 ml. After redissolving the precipitate by placing the bottles in a boiling water-bath they were allowed to stand for 48 hours either at room temperature, in a thermostat at 20° or in the refrigerator according to the temperature at which the precipitability was to be determined. The contents of the bottles were then decanted through a dry filter and the nitrogen in the filtrate determined in aliquots (referred to the original volume of 40 ml.) by the micro-Kjeldahl method. The amount of cystine-N in the precipitate was then calculated by difference.

SUMMARY.

The precipitation of cystine by 24-phosphotungstic acid at varying concentrations of the former has been studied under the usual conditions of protein analysis with the following results.

1. The extent of the precipitation at any particular temperature is dependent on the concentration of cystine present.
2. Below a certain minimum concentration of cystine no precipitation takes place, so that a protein may contain cystine and still show no cystine-S in the phosphotungstic acid precipitate.
3. Precipitation does not appear to be complete at any concentration. At the concentrations of cystine usually present in protein hydrolysates only a small fraction is precipitated. Cystine-N values based upon this method are therefore of no significance.
4. Nearly quantitative precipitation is obtained only at such concentrations or at such low temperatures as are inapplicable in the analysis of proteins.

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CVIII. AUTOXIDATION OF THE FATTY ACIDS.

II. OXIDO-ELAIDIC ACID AND SOME CLEAVAGE PRODUCTS.

By GEORGE WILLIAM ELLIS.

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(Received March 12th, 1936.)

IN Part I [Ellis, 1932] a method for studying the oxygen uptake of the fatty acids was described. Elaidic acid showed an uptake of over 20 % of its weight in about 36 hours. This rate depended upon high dispersion of the acid at temperatures above its melting point, upon its thorough admixture with cobaltous elaidate as a catalyst and upon a circulation of oxygen with the accompanying removal of the carbon dioxide formed. In subsequent experiments this degree of autoxidation (which marks the end of an even rate of oxygen uptake) has been attained in less than 7 hours; partly by increasing the rate of the circulation of oxygen to 2 ml. per sec. as compared with 0.4 ml. per sec. previously employed. A probable explanation of this effect is that the carbon dioxide formed causes a lowering of the partial pressure of oxygen and may even become adsorbed by the dispersed liquid and its products, so retarding the autoxidation unless removed by a rapid passage of oxygen. An increased proportion of the catalyst from 0.05 % of the metal to 0.5 % has also increased the velocity of the reaction, but no further increase by greater concentration has been noted.

The biochemical significance of this work is not lessened by devoting most of the study to pure elaidic acid instead of to oleic acid, which is so difficult to purify. In Part I it was shown that both acids autoxidise at comparable rates, and since examining the products in the two cases, there is little doubt that their differences may be attributed to stereoisomerism. Nor does the employment of a dry film and of dry oxygen gas detract from this significance, for it will be shown that there are close similarities both in the rate and in the products of dry autoxidation as compared with those observed for autoxidation in oxygen saturated with aqueous vapour at the temperatures of autoxidation. The latter observation has been made with a slight modification of the apparatus shown in Fig. 1, Part I. The cylinder A, containing the dispersed acid, was provided at its base with two perforated porcelain discs between which cotton wool soaked in water was placed. The upcoming oxygen was therefore saturated at the temperature of the reaction. It should be noted that water is an immediate product of autoxidation so that working under actual dry conditions is impracticable. The conditions adopted, therefore, may be compared with the autoxidation of an oil emulsion. Olive oil becomes rancid rapidly when in the form of a very fine emulsion and when a metallic catalyst is present, but it is doubtful whether such a process could lead to satisfactory results: the emulsion would have to be very bulky and autoxidation would lead probably to demulsification and so to practical stoppage of the reaction.

The products are numerous and in some cases complex. Reduction in the time of autoxidation does not appear to have simplified the process or to have produced a main, initial autoxidation product. It has been necessary for practical

results to work at temperatures above 55°. The results obtained in recent work are set out in Table I. This Table is a continuation of Table I, Part I. The elaidic acid has been allowed to autoxidise in lots not exceeding 23 g. A single experiment will not yield fractions which suffice to give some of the quantitative results to be described. Over 70 autoxidations have been carried out and similar fractions have been combined for a number of the actual estimations. The various products obtained will be described under different headings below and in further communications. They are given in the order in which they may be isolated.

Carbon dioxide and water.

These are absorbed from the circulating oxygen system as described in Part I, where the results of various estimations were tabulated. A few additional results have been obtained. In Exps. 4, 16, 15 and 10 of Table I the oxygen uptakes are 16.7, 18.2, 21.6 and 21.1 % respectively (col. 8). The oxygen evolved as carbon dioxide was estimated as 4.4, 4.6, 4.9 and 5.2 % respectively, accounting for 25 % of the oxygen absorbed. These results are in agreement with those obtained in Part I. The water evolved was estimated in Exps. 15 and 16 and amounted to 7.0 and 7.5 % as oxygen. The results of water determinations may be too high, owing to the presence in the absorption tubes of volatile acids. A total water and carbon estimation may be obtained from the difference of the total products calculated from the oxygen uptake and those other than water and carbon dioxide actually isolated. Exp. 74 gave 11.3 % by this method. Exp. 15 gave 11.9 % as oxygen by absorption of both products in dry caustic soda. Both results are probably high, but there is little doubt that when elaidic acid shows an oxygen uptake of about 20 %, nearly half this oxygen is evolved as these two products. These results will be referred to again.

The main autoxidation products.

The fine, purified sand, used as dispersing agent, is extracted in a Soxhlet apparatus with ether. From 20 g. of the acid nearly 23 g. of an oily extract are obtained. This mixture is yellow to light brown and will darken slightly on a water-bath. Otherwise it appears fairly stable. Kept for some hours it will deposit crystalline matter, which cannot be separated advantageously at this stage. It is completely soluble in sodium carbonate and titrates to an equiv. wt. about 250. It gives i.v. up to 50, which is partly accounted for by the presence of unchanged elaidic acid. It possesses a pungent odour resembling "drying" linseed oil. This odour is also obtained in the autoxidation of stearic acid (Part I). It liberates iodine from an acetic acid solution of potassium iodide in amounts corresponding to 1–2 % of a peroxide of elaidic acid. When boiled with water or dilute acids, the aqueous portion gives a faint colour with titanous acid, but not more than can be accounted for by the peroxides in the ether used in the extraction. It is miscible with small proportions of most solvents, but is only partly soluble in large proportions of light petroleum, carbon disulphide and cold methyl alcohol. It forms a slightly gelatinous solution in benzene. Many attempted fractionations of the salts of lead, zinc, magnesium, calcium and copper have not proved satisfactory. Of the various methods tried, that which has proved the most successful for the separation of the various constituents will be detailed under the different headings below. It has been found advisable to express their yields as the amounts obtained from the autoxidation of 100 g. of elaidic acid. For this purpose, when necessary, any unchanged acid has been estimated and this amount deducted from the weight experimented upon.

Table I. *Products of the autoxidation of elaidic acid.*

Percentages relate to 100 g. of elaidic acid autoxidised.

No. of exp.	Weights taken g.	Weights autoxidised g.*	Duration hrs.	Temp. °C.	Catalyst % by wt. as at N.T.P. †	Oxygen total uptake by N.T.P. ‡	Azelaic and suberic acids % ‡	Nonanoic and octanoic acids % §	Oxido-elaidic acid % ¶	Oil sol. petroleum products % ¶	Other oily products % ¶	Remarks
1	5.24	5.24	67	70	0.4	1088	29.7	(11.0)	—	—	10.4	40.0
2	4.60	4.60	26	55	0.2	553	16.6	—	—	—	—	Oxygen saturated with aqueous vapour
3	20.00	17.52	43	65	Nil	2357	19.2	8.5	(3.8)	—	—	—
4	7.00	6.55	48	65	Nil	765	16.7	(10.0)	(5.3)	28.2	37.2	Oxygen saturated with aqueous vapour
5	20.18	18.52	19	63	0.1	2194	17.2	(10.8)	—	14.4	26.8	—
6	20.18	16.35	19	63	0.1	2240	19.6	—	—	16.3	25.4	46.1
7	20.91	17.29	48	50	0.4	1614	13.4	—	—	—	77.9	—
8	20.91	13.88	30	35	0.4	2313	23.8	(6.3)	5.0	32.3	43.8	—
9	22.98	18.15	10	65	1.3	2700	21.3	(15.3)	—	16.8	32.7	35.4
10	20.91	20.64	7	78	0.4	3054	21.1	(10.0)	6.7	11.7	27.1	38.7
11	20.00	17.72	48	75	Nil	2368	19.1	9.1	—	1.6	23.5	39.6
12	21.81	21.80	17	68	0.9	3279	21.0	9.3	8.1	15.1	30.5	36.7
13	21.37	16.09	23	66	0.9	1741	15.6	—	(9.2)	0.4	17.2	44.4
14	21.81	18.32	10	64	0.9	2550	19.9	(11.0)	—	14.8	—	30.8
15	7.45	6.74	12	67	0.7	1017	21.6	(11.4)	(4.9)	17.4	26.7	35.8
16	5.00	3.06	22	76	Nil	390	18.2	(6.5)	(3.1)	2.7	19.9	51.5
17	7.45	6.00	6	67	0.7	798	19.0	(9.4)	(3.1)	12.2	22.8	48.9
18	4.91	3.91	7	72	1.8	706	25.8	(11.0)	—	10.1	34.4	30.9
19	4.91	4.26	29	57	1.8	868	28.5	(6.5)	—	15.1	28.6	34.8

* I.e. col. 2 less acid recovered unchanged.

† Present as cobaltous elaidate.

‡ Calculated as azelaic. Results in brackets are approximate.

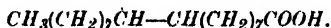
§ Results in brackets are approximate.

¶ Results include those of col. 10.

¶ To be described with previous column in another communication.

(Oxygen contained aqueous and hydrochloric acid vapours)

Oxygen saturated with aqueous vapour. Duration continued 21 hrs. at 50°

The product oxido-elaidic acid with notes on its isomeride oxido-oleic acid.

The main autoxidation products from elaidic acid, obtained as described above, were dissolved in seven times their bulk of alcohol. The solution was cooled in ice and diluted with water until a cloudiness appeared. Salt was then added to the ice and the solution of the products stirred from time to time during several hours. The deposit of crystals was filtered rapidly on a cooled Büchner funnel and washed with a little cold 70% alcohol. A further small crop of crystals has sometimes been obtained by cooling the mother-liquor by means of carbon dioxide snow. The dried crystals were fractionated in light petroleum. The solution must not be cooled below 15° until most of the oxido-elaidic acid has been separated: otherwise unchanged elaidic acid is deposited and little fractionation is effected. The oxido-elaidic acid separated in this way was recrystallised from dilute alcohol or acetone. It is readily soluble in most solvents in the cold, with the exception of light petroleum. It crystallises from alcohol, acetone or light petroleum in minute but perfectly formed rhombic plates of m.p. 55.5°. (Found: equiv. wt. 299; mol. wt. cryoscopic in benzene 333; in acetic acid 285; by Rast's method 304. C, 72.48; H, 11.35%. $\text{C}_{18}\text{H}_{34}\text{O}_3$ requires mol. wt. 298; C, 72.42; H, 11.49%.) It gave i.v. 1.0 and 0.0. When boiled with 40% NaOH it was nearly completely converted into dihydroxystearic acid, m.p. 133°. An identical oxido-elaidic acid was synthesised. Elaidic acid was dissolved in dilute NaOH and 1½ mol. proportions of dilute NaOCl added. Carbon dioxide was passed in and the mixture left for 12 hours. The solid chlorohydrin was separated, dried and added to excess of sodium ethoxide in alcohol. The mixture was shaken frequently for 2 hours at about 40°. The acidified, diluted solution gave a good yield of crystals, which on purifying with light petroleum gave the same minute rhombic plates melting at 55.5°, unchanged on admixture with the autoxidation product.

The percentage yields of oxido-elaidic acid are shown in Table I, col. 11. These are for the purified product and are therefore low, but it is not thought that more than 20% of this product is formed. It results from catalytic autoxidation, for Exps. 11 and 16 gave very low results and these are the only two conducted in the absence of the catalyst. The results of Exps. 8 and 13 are the only others giving low figures. The former was conducted at a temperature below the melting point of elaidic acid and was therefore considerably prolonged, whilst the latter represented an autoxidation in the presence of hydrochloric acid vapour. In both these cases hydrolysis of the oxido-derivative must have taken place, but hydrolysis by aqueous vapour for the shorter periods corresponding to those of the majority of the autoxidations is very slight. Exp. 17 gave a fair result although the oxygen employed was saturated by aqueous vapour at the temperature of autoxidation. Further evidence regarding hydrolysis was obtained as a result of Exp. 9, not recorded in detail in Table I. The usual drying tubes were employed with the circulating oxygen, but the contents of the autoxidation cylinder were removed in three parts and examined separately for their oxido-content. These parts comprised a lower portion into which the dry oxygen entered, a middle portion receiving in addition the water vapour formed in the lower portion, and an upper portion under additional humid conditions. These fractions contained 15.6, 13.0 and 9.4% respectively, of oxido-elaidic acid. There is evidence of slight hydrolysis. On the other hand when 1.005 g. of the pure

product were dispersed on sand and maintained at 65° while oxygen saturated by aqueous vapour at this temperature was circulated for 12½ hours, 0.9781 g. of pure oxido-elaidic acid and 0.0136 g. of the nearly pure acid were recovered. There was little or no hydrolysis. Interest in this question was partly due to the possibility of this derivative being a precursor of dihydroxystearic acid. This latter compound is not present in the free state in any appreciable amount as an autoxidation product, but, as will be shown in a further communication, in combination it represents a large proportion of the oily products.

Some interesting comparative work on the autoxidation of oleic acid has been undertaken. This will be described in more detail in a further communication, but mention may be made here of the possible formation of an isomeric oxido-oleic acid. Pure oleic acid was not employed, but the commercial samples were nearly freed from the saturated fatty acids by crystallising these out from dilute acetone at -30°. The crystalline portion of the autoxidation products was very soluble and could only be separated from dilute alcohol by cooling well below 0° for some hours. Recrystallised from light petroleum, a fraction only slightly soluble in this cold solvent yielded the thin, minute, rhombic crystals of oxido-elaidic acid. The melting point of this and of its mixture with the elaidic acid derivative was 55.5°. The mother-liquors yielded a crystalline mass of m.p. 52° and this was thought to be the oxido-oleic acid obtained by Bauer and Bahr [1929] by the oxidation of oleic acid with perbenzoic acid and having this melting point. The microscopic appearance of the autoxidation product did not point to its being a pure substance. On repeated recrystallisation from light petroleum followed by dilute acetone a very soluble mass crystallising in long thin plates was obtained. It melted at about 58° but could not be freed from a trace of impurity. It approximately agreed with a perbenzoic acid oxidation product of oleic acid obtained by Pigulevski and Petrova [1926] who gave the m.p. as 57.5-58°. A product synthesised by Nicolet and Poulter [1930] through the chlorohydrin was described as melting at 53.8°, and a similar product from elaidic acid likewise melted at 53.8°. These latter results cannot be accepted. Previously Nach and Saytzeff [1892] had obtained by the action of silver oxide on the dibromide of oleic acid a "glycidic acid" of m.p. 57-60°, while Albitzki [1900] describes a chlorohydrin synthesis of the oxido-derivative of oleic acid leading to an oil, the product from elaidic acid having m.p. 57-60°. In view of the uncertainty existing it was decided to repeat the above described successful synthesis of oxido-elaidic acid substituting oleic acid of fair commercial purity. The yield was poor, nor could the mixture of the small amount of oxido-oleic acid be separated from the saturated fats originally present in the oleic acid. The experiment was repeated with 5 g. of free acid suspended in 500 ml. of water containing a little gum arabic. To the emulsion was added dilute NaOCl (1.5 mols.) and carbon dioxide was passed in until the mixture was saturated. After 12 hours the liquid or mucilaginous chlorohydrin was extracted with ether from the acidified solution. The evaporated solution was dissolved in alcohol and cooled in ice. Water was then added until a slight opacity was observed. The solution was cooled by carbon dioxide snow with constant stirring, and the separated fatty acids were filtered off, advantage being taken of the highly soluble, liquid chlorohydrin for the removal of these impurities. The alcohol solution was dried by anhydrous sodium sulphate and left for 3 hours with excess of sodium ethoxide in alcohol. The diluted, acidified mixture was extracted with ether. A white crystalline mass (25%) was obtained. A comparative treatment of 20 g. of oleic acid yielded 20%. Pure oxido-oleic acid is difficult to isolate. Its solubility may be compared with that of palmitic

acid, with which it was probably mixed. The mixture was fractionated many times with ice-cold light petroleum (40–60°) with the occasional use of activated charcoal in small quantities. The melting point was gradually raised from below 53 to 57°. Finally, the product was recrystallised slowly from about 80% acetone, until it had m.p. 59.5°. The pure product is highly characteristic. It is very soluble in all the common organic solvents including the various chloro-derivatives in use. It crystallises in long plates, so thin as to be almost invisible under the microscope and when suspended in dilute acetone. (Equiv. wt. 296.5; C, 72.50; H, 11.50%.) Boiled with 20% aqueous NaOH it yielded over 90% of dihydroxystearic acid, m.p. 94°; confirmed by a mixed melting point with a known permanganate oxidation product of elaidic acid. Although melting higher than the product obtained by Pigulevski and Petrova [1926] the comparison is only a question of relative purity, for these authors noted hydrolysis to the dihydroxystearic acid of m.p. 94°. It was somewhat unexpected to find the oleic acid product of higher melting point than that obtained from elaidic acid. The readjustment of this reversion on hydrolysis of the oxido-compounds to their dihydroxystearic acids is also of interest.

The total solid acids of the main autoxidation products from 20 g. of oleic acid yielded 3.9 g., composed largely of the oxido-elaidic acid. Its isomeride could not be freed from traces of fatty acids, so that the melting point was not raised to that of the chlorohydrin synthetic product. The characteristic long, thin plates readily soluble in warm light petroleum were obtained. The extreme difficulty of its separation from the mixed fatty acids originally present in the oleic acid accounts for the various failures on the part of previous workers, as mentioned above, to isolate this compound in a pure state. It may be concluded that autoxidation of both elaidic and oleic acids yields the same oxido-elaidic acid, to the extent of about 20%. Oxido-oleic acid does not appear to be formed in any appreciable amounts.

The water-soluble cleavage products: suberic, azelaic and oxalic acids.

The alcoholic filtrate from the crystalline fraction described above is diluted with water until oil is no longer precipitated. The emulsified oil is partly removed by filtration through a fine paper. The filtrate contains for the most part suberic and azelaic acids, which are highly soluble in the diluted alcohol, and which are therefore almost entirely removed in this process from the remaining oily products. This removal is of importance in order to facilitate examination of the oils to be described later. To isolate the dibasic acids the dilute alcohol is evaporated. The residue is taken up in hot water and some oil is removed by absorption on cotton wool. This oil is extracted with hot water until it is clear when cold. The aqueous extracts are concentrated and cooled in ice. The mixed acids are filtered off, dried and treated with chloroform. Most of the suberic acid remains undissolved. The use of this solvent for the separation of suberic and azelaic acids is more efficacious than other methods which have been proposed from time to time. The suberic acid is purified from chloroform containing a little alcohol. Very pure products have been obtained, possessing the characteristic crystalline appearance and solubilities. One preparation gave: m.p. 140°, equiv. wt. 88.4, 88.8. A mixed melting point established its identity. Azelaic acid has been isolated in the pure condition with difficulty. The mother-liquors were evaporated, and the crude acid crystallised from concentrated, warm aqueous solution. Traces of suberic acid in the chloroform mother-liquors accumulate with the azelaic acid at this stage, so that further treatment with chloroform becomes necessary. The azelaic acid is then crystallised from benzene and

finally again from water. This acid has been obtained in a pure form from a number of experiments. It possessed the highly characteristic crystalline appearance, forming shining, rectangular platelets. It gave M.P. 106° , equiv. wt. 94.0, 94.5, and a mixed M.P. confirmed its identity.

Quantitative work involved in the separation of these acids must be approximate only, but it was noticed in one separation that whilst about 0.57 g. of fairly pure suberic acid was isolated, only 0.45 g. of azelaic acid was obtained. The latter would suffer greater loss owing to its more difficult isolation, but the large proportion of the 8-carbon dibasic acid indicated a cleavage of some interest, particularly as no other dibasic acid could be isolated. For this reason a further product comprising these mixed acids was thoroughly investigated. Many fractionations on the lines indicated above were conducted which need not be recorded. Adipic, pimelic and sebacic acids were carefully looked for. It is doubtful whether they are present even in traces. The results obtained were as follows: pure suberic acid 1.27 g.: pure azelaic acid 0.74 g.: impure azelaic acid (M.P. $86-104^{\circ}$, equiv. wt. 111) 0.06 g., residue (impure, white solid) 0.23 g., equiv. wt. 106. Further separation could not be effected. These results show almost certainly that the cleavage of this form of oxidation is almost entirely such as gives rise to azelaic acid and to suberic acid. The isolation of the latter led to a search for oxalic acid. This could not be found with these acids. Its low solubility in ether, together with its probable combination with the cobalt used as catalyst, tends to prevent its removal in the extract of the sand described above. In several cases this sand was subsequently extracted with dilute HCl. This, on evaporation to small bulk, neutralisation and precipitation of the cobalt as sulphide, yielded a calcium salt insoluble in acetic acid. Permanganate titrations of these salts treated with sulphuric acid gave amounts varying from a trace to 0.15 %.

The percentage yields of the mixture of azelaic and suberic acids are set out in Table I, col. 9. These figures are too high in most cases because the diluted, alcoholic solution yields on evaporation some oil which is not redissolved in pure water. The figures in brackets are obtained by titration of the solution of the crude acids before the oil is removed, and the titration value has been expressed as azelaic acid. In the several cases in which the crude acids have been analysed for azelaic and suberic acids as described above, the yield of oil has approximated to 20 or even to 30 %. On the average, deducting 20 % of oil, about 8 % of these acids has been obtained, and this, as shown, represents about 4 % of each of the two acids. Azelaic acid is of almost common occurrence amongst the products of any oxidation of oleic or elaidic acid. The appearance of suberic acid, with traces of oxalic acid, will be referred to below.

The volatile cleavage products: nonanoic and octanoic acids.

The oily products precipitated from the diluted alcohol as described above are taken up in light petroleum ($40-60^{\circ}$). They are miscible with small proportions of this solvent but continue to be precipitated almost indefinitely on dilution. In practice these products, when from 20 g. of elaidic acid, have been extracted with about 400 ml. of the ice-cold solvent. On evaporation a clear, straw-coloured or almost colourless oil remains. Some of the yields of this fraction are shown in Table I, col. 12. The variations (20-30 %) are partly due to the imperfect separation of this fraction from that of the insoluble fractions shown in col. 13. Nonanoic and octanoic acids have been obtained from the light petroleum fraction by steam-distillation and also by distillation *in vacuo*. These acids have also been obtained by steam-distillation of the whole autoxidation

products, in which case they may be accompanied by nonaldehyde due to a decomposition to be dealt with in a further communication. The two volatile acids have been isolated and identified in the course of several different experiments. In one case the zinc salts were obtained from a steam-distillation product. Crystallisation from alcohol yielded fairly pure zinc nonanoate, M.P. 129°. The acid recovered was of the highly characteristic odour; it readily crystallised in ice and had equiv. wt. 159. When the mixed volatile acids from several experiments were fractionally distilled *in vacuo* the first fraction had equiv. wt. 149. This is a little high for octanoic acid (144), probably owing to the presence of a little nonanoic acid. Moreover the not unpleasant odour of nonanoic acid is markedly different from the sweat-like odour of octanoic acid as obtained in the first distilled fraction. This product likewise crystallised readily on cooling. It would not be possible to separate quantitatively these two acids. Their presence was to be expected when it was realised that azelaic and suberic acids were amongst the products of autoxidation. Since the dibasic acids appear to be formed in about equal amounts the two volatile acids should be formed to the extent of 83 % of these, assuming mol. proportions of azelaic and nonanoic and of suberic and octanoic acids. Actually as will be seen by reference to Table I, col. 10, the yields obtained are considerably less, but the volatile acids are more difficult to determine quantitatively. They are slightly soluble in the diluted alcohol which is employed to remove the dibasic acids. Further, their vapour pressures at the temperatures of autoxidation are sufficient to cause their appearance in the absorption tubes of the oxygen circulating system employed. The latter cause for low results particularly accounts for those of Exps. 10 and 17, when small quantities of elaidic acid were experimented upon, and where the error becomes more apparent. Generally the results shown in cols. 9 and 10 are confirmatory and point to a total of 16–20 % of these cleavage products from the elaidic acid autoxidised.

The smallness of the amount of oxalic acid obtained is due, with little doubt, to its suffering further autoxidation, for Jorissen [1899] has shown that solutions of this acid autoxidise in the presence of magnesium sulphate, a catalyst closely comparable in its effect with the salts of cobalt. Curiously, Jorissen observed that the absence of light was a necessary factor, which, however, was a condition of the present experiments. This cleavage is comparable with the oxidation of oleic acid by potassium permanganate in slightly alkaline solutions. Lapworth and Mottram [1925] showed that suberic, oxalic and octanoic acids are obtained in nearly theoretical yields when dihydroxystearic acid M.P. 133° is so oxidised. The latter is formed from oleic acid by the same reagents under more carefully controlled conditions. It may be a precursor of the three cleavage products. Mention has been made that this dihydroxystearic acid occurs in combination amongst the oily products to be described. Saytzeff [1885], however, showed that a similar permanganate oxidation of elaidic acid gave the dihydroxystearic acid M.P. 94°, which has not been found as an autoxidation product. Although selective oxidation may tend to produce only one of the two dihydroxystearic acids, either may suffer cleavage of the nature found in this autoxidation.

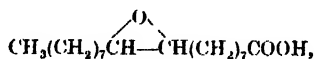
The disappearance of most of the oxalic acid accounts for about half of the carbon dioxide obtained during these autoxidations, but only for a small proportion of the water formed.

SUMMARY.

The rate of autoxidation of elaidic acid has been increased from that described in Part I to an oxygen uptake of 20 % in less than 7 hours. Carbon dioxide must be removed by rapid circulation of oxygen over the dispersed acid, and as much as 0.2 % of cobalt, as the salt of elaidic acid, must be added. The presence of aqueous vapour does not appreciably affect the rate of autoxidation.

When a 20 % oxygen uptake is recorded, a large proportion of this oxygen is evolved as carbon dioxide and water. The former may account for 25 % of this oxygen and the latter for even more.

Oxido-elaidic acid,



is formed during catalytic autoxidation of elaidic acid to the extent of 16 or perhaps 20 % of the acid autoxidised. It is only formed in small amounts in the absence of a catalyst and can be obtained in nearly similar amounts whether autoxidation occurs in oxygen dried by calcium chloride or saturated with aqueous vapour. It melts at 55.5° and crystallises in minute, perfectly formed rhombic plates. It is fairly stable and requires strong alkali for hydrolysis to dihydroxystearic acid m.p. 133°. It is not readily further acted upon by oxygen in the presence of cobalt.

An identical oxido-elaidic acid has been synthesised. Similar autoxidation of oleic acid of fair purity yields about 20 % of the same oxido-elaidic acid but in addition a small percentage of an oxido-oleic acid of m.p. 59.5° which crystallises in very thin elongated plates, and which has been synthesised from the chlorohydrin of oleic acid. Both of these isomerides, obtained by autoxidation and by syntheses, have been purified with great care with a view to clearing up some doubts which had arisen regarding various syntheses of these compounds previously described by different workers.

Certain cleavage products obtained amount to 16–20 % of the elaidic acid autoxidised. Of these the dibasic acids consist almost entirely of azelaic and suberic acids in about equal amounts and of a trace of oxalic acid. Of the volatile acids nonanoic and octanoic acids predominate. In addition to the very usual cleavage of elaidic acid into azelaic and nonanoic acids, there appears to be one causing a simultaneous double fission into suberic, oxalic and suberic acids. This latter seems to approximate in amount to the former.

With an oxygen uptake of 20 % the 120 % products obtained from 100 parts of elaidic acid include: carbon dioxide about 6.5 %; oxido-elaidic acid 16–20 %; cleavage products azelaic, suberic, oxalic, nonanoic and octanoic acids totalling 16–20 %. These account for 42–54 %. The remaining 68–76 % are the oils which will be described in a further communication.

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CIX. STUDIES ON BILE PIGMENTS.

II. A NEW TEST FOR BILIRUBIN IN THE URINE AND ITS USE FOR DETECTION OF BILIRUBIN IN NORMAL URINE.

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Tests for bilirubin in the urine are unsatisfactory owing to their lack of sensitivity. Gmelin's test with nitric acid and Rosin's test with iodine, depending on the formation of green biliverdin or blue bilicyanin, are positive only in the presence of high concentrations of pigment and many modifications of these methods do not give much better results. The reason for this failure is the fact that biliverdin or bilicyanin is only a transitional stage of bilirubin oxidation, the final stage being represented by the pink choletein which is formed more rapidly in the presence of smaller amounts of bilirubin. Yet it is just in cases of slight jaundice that the detection of small amounts of bilirubin is of particular diagnostic importance.

Zins [1923] modified Steensma's method by adsorbing the pigment on BaSO_4 and testing the precipitate on the filter-paper with trichloroacetic acid. Cole [1926] described a modification of Huppert's method, adsorbing bilirubin on BaSO_4 , eluting the pigment with acid alcohol and oxidising it with KIO_3 . Kuhn [1928] performs the oxidation by an alkaline copper solution and extracts the biliverdin in a layer of alcohol on the top. A spectroscopical test after oxidation of bilirubin to bilicyanin is described by Beccari [1928]. Italic [1929] devised a modification of Steensma's method, adsorbing the pigment on talc, eluting it with acid alcohol and oxidising it with NaNO_3 . Recently Godfried [1934] described a test devised by Harrison who adsorbs the pigment on BaSO_4 and tests the precipitate on the filter with a drop of Fouché's reagent.

EXPERIMENTAL.

Preliminary experiments showed that bilirubin is most efficiently adsorbed by filtering through a layer of adsorbing material. The urinary pigments being adsorbed on the surface of the layer the bilirubin can then easily be detected with an oxidising reagent. Talc was found to be the most suitable adsorbent and for oxidation Fouché's reagent or nitric acid are equally suitable. The test is performed in the following way:

A Büchner funnel of $3\frac{1}{2}$ cm. diameter is fitted with the ordinary filter of 3 cm. diameter which is wetted with water. 5 ml. of 10% talc suspension in water after shaking well are poured on the filter and, after sucking dry, 5 ml. of urine are poured on the talc layer, which, after sucking off again appears as a yellow or orange disc. One drop of Fouché's reagent¹ or 10% HNO_3 is put in the middle of the talc disc and sucked off. Even traces of bilirubin are indicated immediately by a distinct blue spot. The colour intensity increases for 1-2 hours fading slowly until, usually after 20 hours, only a faint grey is perceptible unless the reaction has been very strong.

¹ Fouché's reagent consists of 25 g. trichloroacetic acid, 10 ml. of FeCl_3 10% and 100 ml. distilled water.

The method of adsorption by filtration has advantages over the usual method in which a precipitate is formed in the urine; the bilirubin remains on the uppermost layer and so produces favourable conditions for the test. The difference between the two methods is illustrated by the following experiment.

5 ml. of 10% talc suspension are filtered as described above and 5 ml. of urine containing a slight excess of bilirubin are poured on the talc layer. Another portion of 5 ml. of urine is shaken with 1 g. of talc after acidifying slightly with acetic acid and filtered off. The first talc layer will be found much more stained than the second and if a drop of Fouché's reagent is put on both the blue spot on the first filter is much stronger than on the second.

It is furthermore essential to use an acid-resistant adsorbent; most of the adsorbing substances such as CaCO_3 , BaPO_3 , BaCO_3 , CaPO_3 , etc., are partly dissolved by acid reagents, so that part of the pigment is dissolved and therefore subjected to a much more vigorous oxidation and transformed rapidly into choletelin. The reaction between acid and adsorbed pigment, on the other hand, is milder and can be better controlled. Thus, by using oxidising agents of different strengths it is possible to produce at will any transitional stage of oxidation between bilirubin and choletelin from yellow through green, blue, violet to pink. Table I gives a survey of these colour reactions with different reagents. As will

Table I. *Tests with drops of different oxidising agents on a talc disc with adsorbed jaundice urine.*

Reagents	Colour of reaction
Fouché's reagent	Blue
HNO_3 95%	Pink
HNO_3 25%	Violet
HNO_3 10%	Blue
HNO_3 5%	Bluish green
HNO_3 1%	Green
Trichloroacetic acid 50%	Greyish white
Trichloroacetic acid 25%	Grey
Obermeyer's reagent	Brown
KMnO_4 N/10	Pink with greenish periphery
Iodine N/10	Brown with greenish periphery
H_2O_2 90/100 vol.	No colour reaction
H_2O_2 90/100 vol. + FeCl_2 10%	Pink with sharp blue ring

be seen Fouché's reagent and 10% HNO_3 are the most suitable for producing a blue bilicyanin reaction. More concentrated HNO_3 as well as KMnO_4 and iodine have more vigorous actions, oxidising the bilirubin rapidly to choletelin. H_2O_2 reacts only in conjunction with FeCl_2 producing a spot with a centre of pink choletelin surrounded by a circle of blue bilicyanin.

It is interesting to note that the method gives a positive reaction also with normal urine, and as this is not the case with other methods it is necessary to show that this positive test is actually due to bilirubin. The only possible interfering substances would be indole compounds which can be transformed into indigo blue. However, pure indole did not react with Fouché's reagent, and indican only after about 30 min. and neither gave a pink choletelin colour. Furthermore, urines with a large amount of indole as shown by Jolles's test, did not give an increased bilirubin test nor did a positive test appear if before adsorption the urine was treated with strong oxidising agents. The blue reaction in normal urine can therefore be attributed to the presence of bilirubin.

The sensitivity of the test was determined by dilution of pure bilirubin solutions until a faint grey reaction was just observed. Proceeding according to the method of Van den Bergh and Grottepass [1934] the bilirubin was dissolved

finally in diluted aqueous NaOH, diluted alcohol and urine freed from preformed bilirubin by treatment with HNO_3 and subsequent neutralisation. The limiting concentrations varied with the solvents being 6.0, 0.8 and 0.9 parts per million in NaOH, alcohol and urine respectively. These values compare favourably with the value of 70 parts per million in Harrison's test according to Godfried's statement.

The dilution technique may be applied to obtain an approximate figure for the actual concentration of bilirubin in normal urine. From 20 normal urines the limiting dilutions for a positive reaction varied between 1 : 350 and 1 : 480. These figures multiplied by the limiting figure of 0.9 part per million as stated above give a content of roughly 0.3 mg./100 ml., a figure corresponding to the bilirubin content of the serum. Assuming a daily urinary output of $1\frac{1}{2}$ l. the output of bilirubin in normal urine should be about 5 mg. per day. However, these figures cannot be more than approximate.

SUMMARY.

1. A new test for bilirubin in urine has been described consisting in the adsorption of urinary pigments on a layer of talc and the production of blue bilicyanin by oxidation with a drop of Fouché's reagent or 10 % HNO_3 .
2. The described method permits for the first time the detection of bilirubin in normal urine.
3. The blue bilicyanin reaction in normal urine has been investigated with regard to the interference of indole compounds and has been found to be specific for bilirubin; excessive amounts of indican react only after about 30 min.
4. The limits of detectability of pure bilirubin dissolved in weak NaOH, diluted alcohol and urine freed from preformed bilirubin are 6.0, 0.8 and 0.9 parts per million respectively.
5. Quantitative estimation by means of a dilution technique gave a bilirubin content of approximately 0.3 mg./100 ml. in normal urine and an output of about 5 mg. bilirubin per day.

I wish to record my best thanks to Dr Arthur Davies, Director of the Devonport Pathological Laboratories of Seamen's Hospital, Greenwich, for his help and interest in my work, to the Seamen's Hospital Society for their assistance and hospitality to me and my assistant Miss Larissa Frenkel, whose cooperation has been of value and to the Academic Assistance Council for a personal grant.

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CX. PROTEASES AND ONTOGENESIS.

I. CATHEPSIN IN THE CHICK EMBRYO.

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(Received March 17th, 1936.)

THE distribution and activity in the embryo of proteolytic enzymes, especially cathepsin and polypeptidases, have not hitherto attracted very much attention. In a paper on the activity of cathepsin in tumours, Krebs [1931] found only a small amount of cathepsin in chick embryos of 8 days' incubation, the proteolytic factor (PQ) being only 1.24, whilst in the most active tissue (rat kidney) he found PQ 25-30. Similar results have been obtained in a fragmentary way by Maschmann and Helmert [1933], Borger and Peters [1933] and others. The present paper describes a systematic investigation of cathepsin in the chick embryo.

EXPERIMENTAL.

The method of Krebs was applied with small modifications. The chick embryos, after being removed from the eggs, were washed several times with Ringer solution, then weighed and crushed in the mortar with a suitable amount of glycerol (100 ml. 87 % glycerol + 0.5 ml. glacial acetic acid). 2 ml. of glycerol being added to each gram of tissue. The mixture was left for 7 hours at room temperature and then for about 15 hours in the refrigerator at 0°. The extract so obtained was centrifuged and used as the enzyme extract. Gelatin purified by HCN and acetic acid was generally used as substrate, but in some experiments ovalbumin and lecithovitellin were used. To 5 ml. of 8 % gelatin, 1 ml. of enzyme extract was added, with either 1 ml. of 0.8 % cysteine or 1 ml. of water. At the beginning of the experiment and after incubation for 28-33 hours at 37°, the amino-N in 1 ml. of solution was determined by the Van Slyke method.

The activity of cathepsin is expressed as follows [Krebs, 1931]:

$$PQ = \frac{\mu l. N_2}{t v \frac{a}{5(a+b)}},$$

where t = time of action of enzyme, v = vol. of enzyme extract used in the experiment, a and b = vol. of tissue and of glycerol, respectively, used for extraction. Since in this equation the dry weight of tissue is calculated using factor 5 we had to change this in our calculations in order to get the true dry weight, which changes with age of the embryo [Needham, 1931].

In the first experiments the increase of amino-N in solutions of gelatin without enzyme and in corresponding enzyme extracts was also determined, but this increase was so small that it had no influence on the final results. Similar controls, however, were necessary in experiments with enzyme extracts from the yolk-sac.

Incubation of the hen's eggs was carried out in electrical incubators as described by Murray [1925], but the humidity was somewhat below optimum. The eggs were from pure-bred White Leghorn and Rhode Island Red hens.

RESULTS.

The optimum p_H for the cathepsin of the chick embryo agrees with that for cathepsin from other sources (Table I). Cysteine hydrochloride was used as enzymic activator. Oxidation of the cysteine was incomplete (nitroprusside

Table I. *Effect of p_H on activity of chick embryo cathepsin.*

Embryo of 7 days		Embryo of 9 days	
p_H	PQ	p_H	PQ
4.0	0.06	4.0	0.12
4.4	0.16	4.7	0.2
4.6	0.16	4.9	0.18
5.0	0.16		
5.2	0.16		
5.4	0.06		

reaction) even after 33 hours under the experimental conditions at p_H 4.7 and it was not necessary to make the experiments in absence of air. However, a few parallel experiments were performed under aerobic and anaerobic conditions and found the same PQ in both cases, e.g. 0.24.

Table II. *Change of activity of cathepsin during development.*

Days	PQ ₀	Average	PQ ₁₃₄	Average
3½	0.26		0.37	
4	0.27		0.42	
	0.23	0.25	0.48	0.45
5	0.18		—	
	0.23		—	
	0.29	0.24	0.42	0.42
6	0.24	0.24	0.39	0.39
7	0.32		—	
	0.18	0.25	0.38	0.38
9	0.23	0.23	0.34	0.34
11	0.16		0.31	
	0.27	0.22	0.41	0.36
13	0.21	0.21	0.46	0.46
15	0.19	0.19	0.46	0.46
18	0.24		0.59	
	0.35		—	
18/19	0.22	0.27	0.28	0.43

During the whole life of the chick embryo the state of activity of cathepsin undergoes little change (Table II). Between 11 and 15 days' incubation the activity is perhaps a little smaller, but even these changes in PQ are hardly outside the experimental error. The catheptic activity is very small, whether activated by cysteine or not. In agreement with Maschmann we found a great part of the cathepsin in the activated state, this activation depending probably on the presence of —SH groups in the enzyme extract. We could always find a positive nitroprusside reaction in fresh enzyme extract, though sometimes it was very faint. It is interesting that the extent of activation by cysteine is nearly the same in all our experiments and very rarely more than 100%. It seems to indicate that about half of the cathepsin in chick embryo tissues is in the activated state. But it is noteworthy that in our experiments the PQ is even lower than found by Krebs. On testing rat kidney we found a PQ of 16, as against 25–30 in Krebs's experiments. Probably these differences depend on the gelatin used.

Table III. *Activity of cathepsin in the yolk-sac.*

Days	PQ ₀	PQ _{cyst}
3½	2.48	5.34
4	2.57	4.74
5	3.3	6.1
12	3.2	5.8
18/19	2.8	6.8
18/19	3.5	7.2

It is very interesting that the activity of cathepsin in the yolk-sac (Table III) is much greater than in the embryo tissue. The average PQ of yolk-sac is about 3 and when activated by cysteine about 5–6. It seems probable, in view of the great synthetic task of embryonic tissues, that the work of decomposing the proteins takes place, not in the embryo itself, but in the surrounding membranes which send to the embryo the material necessary for building new tissues.

Experiments on the distribution of cathepsin in the embryo were performed by determining the activity of cathepsin in the head part and the tail part of the embryo separately. In both parts we were able to find cathepsin, as shown in Table IV.

Table IV. *Distribution of cathepsin in the embryo.*

	Embryo of 6 days		Embryo of 18 days	
	PQ ₀	PQ _{cyst}	PQ ₀	PQ _{cyst}
Total	0.14	0.29	—	—
Head part	0.07	0.19	0.23	0.24
Tail part	0.26	0.34	0.23	0.46
Liver	—	—	4.7	5.7
Kidney	—	—	2.2	4.2

In the embryo of 6 days the amount of cathepsin in the tail part is much greater than in the head part, but in the embryo of 18 days the PQ in both parts is the same. The apparent change in distribution probably depends on the development in the tail part of the embryo of heavy legs, feathers *etc.*, which do not contain cathepsin. In the liver and kidneys we found a comparatively greater amount of cathepsin, but not as great as in rat kidney.

Lecithovitellin (obtained from egg yolk by the procedure of Blackwood and Wishart [1934]) and ovalbumin were also used as substrates (Table V) as they

Table V. *Experiments with (a) lecithovitellin, (b) ovalbumin.*

Embryo of 9 days.			
	p _H	PQ ₀	PQ _{cyst}
(a)	4.0	0	0
	4.2	0	0
	4.5	0.12	—
	5.8	0.15	0.3
	6.7	0.1	0.18
(b)	4.7	0.24	0.36

seemed a more natural material for study of catheptic activity in the embryo. However, it was not possible to demonstrate any great decomposition of these egg proteins. With lecithovitellin the quotients obtained were even smaller than with gelatin, whilst the p_H optimum appeared to be shifted more to the alkaline side (to p_H 5.8). With ovalbumin in 2.4 % solution, the PQ at p_H 4.7 was nearly the same as with gelatin, *e.g.* 0.24 (or 0.36 with cysteine) in the embryo of 7 days, 0.25 (or 0.36) in the embryo of 18 days.

Results indicating enzymic synthesis of protein under certain experimental conditions have been periodically reported. The importance of certain factors has been deduced: *e.g.* sufficient initial concentration of the products of decomposition [Wasteney and Borsook, 1930], and some connection with oxidoreductive processes [Rondoni and Pozzi, 1933]. Besides this work on "plastein", work on the reversibility of cathepsin action has been described of recent years. It seemed that the embryonic tissue of the chick would be especially good material for such experiments. In such a series of experiments we used the following method. The gelatin (or ovalbumin) was digested by an enzymic extract from yolk-sac for 4-5 days at p_H 4.7, then concentrated and brought to p_H 6-7.2. Then the new enzyme extract from embryo was added. In some experiments we added H_2O_2 , glycogen or $CuSO_4$, substances inhibiting the decomposition of protein. At the beginning and at the end of the experiment amino-N and total protein (Kjeldahl) were determined. In certain cases it was possible to observe the disappearance of amino-N; but on observing all the conditions laid down by the above authors we could not find any synthesis of protein. Further experiments on this very interesting subject will be carried out.

DISCUSSION.

The problem of the utilisation of egg proteins by the embryo is not yet satisfactorily solved. It may be examined in two ways. First, we may examine the disappearance of proteins from white and yolk and their parallel increase in the embryo. Much work has been carried out along these lines, but we do not know accurately in what form the embryo obtains its material for building new tissues—whether in the form of polypeptide complexes or of simple amino-acids. From investigations on tissues growing *in vitro* we can only conclude that the mechanism of nitrogen assimilation is not very simple and it may or may not bear on the problem of the developing embryo.

The experiments of Carrel [1924] and especially of Willmer and Kendal [1932] show the importance of suitable material for this purpose. We have not merely to find the ways and products of decomposition of egg proteins, but also the form under which they are utilised by the embryo. The second line of attack is therefore to examine the enzymes of the whole embryonic system including the membranes. From our present experiments it may be concluded that the proteolytic activity of embryo tissues is very small. Apparently, polypeptidase activity (which, however, has not been examined throughout the whole period of embryonic life) is also not well developed in embryo tissues [Borger and Peters, 1933]. This suggests that decomposition of egg proteins takes place somewhere outside the embryo itself, probably in the yolk-sac. Unfortunately we cannot yet say how far this decomposition extends, or whether the products are "growth proteoses" or amino-acids.

Results showing the beneficial effect of small quantities of embryo extract in the presence of proteoses [Willmer and Kendal, 1932], and our own results seem to us to point to embryonic tissue as a suitable material for examining protein synthesis. But here the question arises as to the character of such synthesis; is it a synthesis of "ancestral" protein (Urprotein) not differentiated from simple amino-acids, as Alcock [1936] would suppose, or rather simply a reversal of cathepsin action as in plastein formation. The theoretical possibilities of such reversibility have been discussed by Borsook [1935], but the need for a source of energy for such synthesis still exists. We have tried to obtain a reversed action of cathepsin, but so far without success.

SUMMARY.

1. The activity of cathepsin in the chick embryo in connection with embryonic synthesis of protein was investigated.
2. The activity of cathepsin in the chick embryo (using gelatin, ovalbumin and lecithovitellin as substrates) is very small whether activated by cysteine or not. With lecithovitellin the p_H optimum is shifted more to the alkaline side.
3. The activity of cathepsin does not undergo much change during the whole developmental period of the chick embryo.
4. The yolk-sac is much more active (15–20 times) than the embryo.
5. No positive results on synthesis were obtained *in vitro*.

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CXI. THE AMINO-GROUPS OF THE PROTEINS OF HUMAN SERUM. ACTION OF FORMALDEHYDE AND NINHYDRIN.

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THE object of the present study was to determine quantitatively the free amino-groups in the proteins of human serum using the Sørensen titration [Obermayer and Wilhelm, 1913], and to investigate the reaction of ninhydrin with proteins. A product of this reaction with definite physical and chemical properties has been isolated.

EXPERIMENTAL.

Preparation of proteins free from amino-acids. 15 ml. of clear human serum were treated with 15 ml. of 10 % trichloroacetic acid. The proteins were separated on the centrifuge, completely redissolved by adding 4-5 ml. of *N* NaOH, to make slightly alkaline (phenolphthalein) and the solution made up to 30 ml. The process was then repeated using 30 ml. of 10 % trichloroacetic acid, and the redissolved proteins were made to 30 ml. Final adjustment to the phenolphthalein end-point was made with *N*/10 HCl or NaOH, with care to keep the protein in solution. Any traces of free amino-acids were found to be completely removed by this treatment.

Formaldehyde titration. 5 ml. of protein solution, adjusted to phenolphthalein as above, were treated with 2 ml. of neutral 35 % formaldehyde and 2 or 3 drops of phenolphthalein. *N*/10 NaOH was then quantitatively introduced until the medium turned reddish again. Subsequent addition of neutral formaldehyde did not change the final alkalinity. The dry weight of protein was simultaneously determined: a 5 ml. aliquot was precipitated with 5 ml. of 10 % trichloroacetic acid, centrifuged and rinsed with distilled water with care to remove no protein; the coagulated proteins were dried at 105° for 24 hours and weighed. It was found (as an average of 20 determinations showing slight individual variations) that a formaldehyde titration amounting to 0.417 ml. *N*/10 NaOH was given for every 100 mg. dry protein. Since each sample was itself a mixture of many individual sera, the result represents an average composition of human serum.

If the formaldehyde-treated protein itself was precipitated and weighed, an increase of weight was always shown. This larger weight however was not a suitable basis for quantitative comparisons.

Sørensen [1907] has shown that the formaldehyde titration is never fully quantitative and has defined conditions such as indicator end-point and formaldehyde concentration on which it depends. Under conditions approximating ours, estimation of amino-acids was about 95 % complete. Recently, Kekwick and Cannan [1936] have emphasised the importance of formaldehyde concentration on the dissociation curve of egg albumin. In our titration, the formaldehyde concentration was greater than 3 *M*, which gave us consistent figures at the phenolphthalein end-point. Much lower figures were obtained for very small concentrations (about 1/20). By applying our routine titration procedure to

pure amino-acids in order to get a comparative empirical figure, we found that 1 ml. of *N*/10 NaOH corresponded to 1.52 mg. of amino-N. From this, it appears that the average human serum contains 0.635 mg. of amino-N per 100 mg. of desiccated proteins.

To discover whether the possible denaturation of proteins by trichloroacetic acid could affect this result, the titration was repeated with proteins purified from amino-acids by ultrafiltration. A mixture of numerous sera was ultra-filtered under a pressure of 20 atmospheres *N* and the separated proteins redissolved in pure water and treated with formaldehyde. Then they were precipitated by trichloroacetic acid and compared with a control precipitate of an untreated portion. From these experiments an average figure of 0.65 mg. of amino-N was found for 100 mg. of desiccated proteins.

Formaldehyde-treated proteins differ from normal proteins in some properties. When once precipitated by trichloroacetic acid, they cannot be brought back into solution again. They are less sensitive to alcoholic precipitation than normal proteins. They do not react with ninhydrin.

Albumins and globulins. A mixture of sera was treated with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ at room temperature. Various fractions of proteins were separated by centrifuging at successive intervals during the following 24 hours. Every fraction was carefully purified from adsorbed $(\text{NH}_4)_2\text{SO}_4$ by repeatedly dissolving and precipitating with trichloroacetic acid, until the filtrate gave no reaction with Ba salts or Nessler reagent.

As an average, proteins collected after a few minutes (globulins) contained about 0.5 mg. amino-N per 100 mg. dry weight, whereas proteins still in solution after 24 hours (albumins) contained 0.85 mg. An actual analysis, including intermediate fractions, is illustrated in Table 1. The amino-N content of the total protein (3.686 mg. per 571 mg. = 0.646 mg. per 100 mg.) closely approximates to that already recorded.

Table 1.

Time of contact with ammonium sulphate	Weight of proteins collected mg.	Amino-N mg.	mg. amino-N per 100 mg. dried protein
6 min.	103.6	0.504	0.486
2 hours	123.8	0.590	0.476
6 hours	38.6	0.152	0.394
24 hours {ppt. solution	131.0	0.760	0.580
	173.8	1.680	0.965
Total	570.8	3.686	—

The action of ninhydrin. To 5 ml. of neutral protein solution freed from amino-acids as described, 0.6 ml. of *N*/10 NaOH and 40 mg. of ninhydrin (dissolved in 2 ml. of water) were added, and the mixture kept stoppered at 37°. In a few hours, some insoluble material (amounting to about 6 mg. per 100 mg. protein and containing small variable traces of nitrogen) appeared and was removed by centrifuging. Thereafter, the mixture remained homogeneous at 37° for many days. Two days after treatment the proteins were precipitated by addition of 1.5 ml. *N*/10 HCl and centrifuged. The supernatant liquor contained no protein, provided that a demonstrable excess of ninhydrin was present. The precipitate was of a dark violet colour and could be redissolved by addition of about 2 ml. *N*/10 NaOH and again reprecipitated by *N*/10 HCl. Treatment with 10 % trichloroacetic acid destroyed the compound.

Ninhydrinised proteins apparently do not react with formaldehyde. When viewed against a strong electric light, a slightly alkaline solution of ninhydrinised

proteins has a reddish colour in presence of phenolphthalein. On addition of $N/10$ HCl, a grey turbidity appears suddenly at the neutral point. Addition of formaldehyde did not seem to produce an increased acidity as usual since no turbidity became visible in a solution of ninhydrinised proteins brought exactly to the neutral point, even though a single drop of $N/10$ HCl was sufficient to produce it immediately after.

Some ninhydrinised protein has been prepared in a greater state of purity, by using ultrafiltration to avoid the use of coagulating agents. Proteins, prepared by ultrafiltration, were treated with ninhydrin as above. It was notable that the usual small precipitate was hardly apparent. Then the whole of the liquid was again ultrafiltered. In the ultrafiltrate an excess of free ninhydrin was demonstrated. The remaining protein was a violet powder which readily dissolved in pure water and gave with formaldehyde no reaction whatever. It was more readily precipitated by HCl from aqueous solutions than were normal proteins.

DISCUSSION.

In this paper, the basis of comparison has been the weight of protein as precipitated by trichloroacetic acid and dried. Such precipitates are heavier than those obtained by addition of two volumes of alcohol or of acetone. The last two are mutually similar and represent, within a somewhat narrow range of variations, about 92% of the trichloroacetic acid precipitates. Although they presumably represent more accurately the actual weight of proteins, they cannot be redissolved in water and so were unsuited to our purpose. If reference to such alcohol or acetone precipitates is desired, the results quoted throughout should be multiplied by 1.085.

The significance of the amino-N estimation is of course restricted by the ordinary limitations of a Sørensen titration. Only amino-groups and the imino-groups of proline and hydroxyproline are ordinarily determined. It may be remarked that the total N of a solution of proteins is not altered by reaction with either formaldehyde or ninhydrin.

The difference in amino-N content of successive fractions of the serum proteins suggests that amino-N determinations might assist in the purification of proteins.

The modified proteins here described might be interesting material for studying the effect of blockage of amino-N on the antigenic behaviour of a protein. An immunological study of these proteins is now being carried out.

SUMMARY.

A quantitative study of the amino-N in the various proteins of human serum by formaldehyde titration is reported. Albumin contains more amino-N than globulins.

The existence of definite reaction products of ninhydrin with proteins from human serum is established. Purification and properties of these products are described.

The physical properties of proteins are considerably altered by combination with formaldehyde or with ninhydrin. The ninhydrin-protein compound no longer reacts with formaldehyde, and *vice versa*.

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CXII. THE IONIC EQUILIBRIUM BETWEEN THE AQUEOUS HUMOUR AND BLOOD PLASMA OF CATS.

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THE origin of the aqueous humour and the nature of the equilibrium existing between it and the blood plasma are problems of considerable significance in relation to the cause of glaucoma. The view that it is the secretion of "ciliary glands" has been gradually abandoned in recent years in favour of the thesis that it is a dialysate from the plasma in the capillaries similar to the cerebro-spinal and glomerular fluids. This theory derives its main support from analyses of the blood and aqueous humour of the horse by Duke-Elder [1927] who found an excess of cations in the plasma over those in the aqueous humour and the reverse for anions in accordance with expectation on the basis of the Donnan equilibrium: diffusible non-electrolytes were approximately equally distributed. However, recent work has cast doubt on the validity of the dialysis theory. Thus Walker [1933] found that (a) the inorganic P concentrations of the aqueous humours of all species examined averaged 50% of those in the corresponding plasmas, (b) in rabbits, dog and man the urea concentration was 68% of the plasma value, (c) the uric acid concentration in fowls and man was 66% of the plasma value and (d) reducing substances were generally present in lower concentration than in the plasma. Adler [1933] showed that in cats the concentration of urea in the aqueous humour was always less than in the plasma. Both Walker and Adler made their determinations on the blood and aqueous humour of the same animal, whereas Duke-Elder carried out a complete analysis of a pooled specimen of aqueous humour derived from the eyes of some thousand horses and compared the results with those of a similar analysis of a single sample of blood obtained from the M.R.C. farm. The significance of Duke-Elder's results depends therefore on the limits of variability from individual to individual of the concentrations of the substances determined in the blood and aqueous humour. In the light of this consideration and the claims of Walker and Adler that the capillary membrane separating the aqueous humour from the blood plasma shows a selectivity incompatible with the assumption that it is an inert membrane, we have carried out careful and accurate analyses of Na^+ , K^+ and Cl^- in the aqueous humours and bloods of cats. The values for any given aqueous humour were compared with those of its corresponding plasma. The results obtained demonstrate that so far as these ions are concerned there is a Donnan equilibrium between the aqueous humour and the blood plasma.

EXPERIMENTAL.

Cats were anaesthetised with Et_2O and bled from the carotid; during the bleeding the aqueous humour was removed from both eyes with a syringe. After clotting, the serum was removed and 5 ml. were measured into a centrifuge-tube. Proteins were removed with trichloroacetic acid, the washings evaporated to dryness in a silica flask and the residue was ashed with conc. HNO_3 [Kutz,

1931]. The ashed residue was made up to 10 ml. with distilled water and used for the Na^+ and K^+ determinations. For determinations of Na^+ and K^+ in the aqueous humour, 0.5 ml. samples were measured into silica flasks and ashed directly with HNO_3 . The ashed residues were washed out into silica beakers, evaporated to dryness and taken up with 1 ml. of water. Na^+ was determined by the method of Barber and Kolthoff [1928], K^+ by Kramer's method [1920] and Cl^- directly on the serum and aqueous humour by a modification of the method of Van Slyke and Sendroy [1923]. The modification consisted in filtering off the AgCl precipitate through an asbestos filter before titration with KCNS . All methods gave errors of rather less than 1 %.

RESULTS.

In Table I are shown the results of experiments on six individual cats (Exps. 1-6) and on the pooled aqueous humours and plasmas of four cats (Exp. 7). In this latter case the individual samples of plasma and aqueous humour

Table I.

Exp. no.	Na			K			Cl		
	Aqueous humour	Serum	R_{Na}	Aqueous humour	Serum	R_{K}	Aqueous humour	Serum	R_{Cl}
1	155	161	1.04	—	—	—	130	124	0.96
2	153	157	1.03	6.25	6.50	1.04	128	123	0.96
3	147	161	1.09	—	—	—	122	117	0.96
4	162	170	1.05	6.20	6.05	0.97	135	135	1.00
5	—	—	—	6.30	6.80	1.08	129	122	0.95
6	151	161	1.07	6.15	6.75	1.10	127	123	0.97
7	150	160	1.05	6.00	6.50	1.08	129	123	0.95

Concentrations are expressed in millimols. per kg. of water.

were pooled in proportion. This rendered possible the use of 5 ml. samples of aqueous humour for ashing just as with plasma so that triplicate determinations of all three ions could be made; although trichloroacetic acid was unnecessary in the case of the aqueous humour, it was added so as to make the conditions identical. In the table the R values indicate the ratios of the concentrations of the ions, expressed as millimols. per kg. of H_2O , in the serum and aqueous humour; thus R_{Na} equals $[\text{Na}]_{\text{S}}/[\text{Na}]_{\text{Aq}}$. Blanks in the table indicate that insufficient material was obtained for all three analyses.

It is evident that in all cases R_{Na} is greater than unity as would be expected on the basis of the Donnan equilibrium; in all cases but one R_{Cl} is less than unity; the values of R_{K} were more variable than the others but similar in trend to those of R_{Na} . The values for Exp. 7 represent the pooled serum and aqueous humour of four cats with a considerably smaller margin of error in the actual determinations.

DISCUSSION.

Van Slyke [1926] has shown that human plasma in equilibrium with its dialysate should give values for R_{Na} and R_{K} of 1.04 and for R_{Cl} 0.96; the experimental values obtained show a fairly satisfactory agreement with expectation. Thus Exp. 7, which is the most significant of all, gave values for R_{Na} and R_{K} of 1.05 and 1.08 respectively and for R_{Cl} 0.95. However, the variability of the values of R as seen in Exps. 1-6, especially of R_{K} , cannot be attributed to experimental errors in the determination of the ions. It must be emphasised that the Donnan ratios should be expressed in terms of activities and there seems no reason to suppose that the activity coefficients will be the same in

both aqueous humour and serum; furthermore the procedure for obtaining the fluids, anaesthesia, insertion of a hypodermic needle through the tough cornea *etc.* must have temporary influences on the local concentrations of these ions; for example D'Silva [1934] has shown that adrenaline, which is liberated during the anaesthetising process, causes an increased blood K^+ . Thus it appears possible that the variations are primarily artificial, so that the assumption that the membrane separating the aqueous humour and the blood plasma is normally capable of selective accumulation of ions is unwarrantable on the basis of the experimental results described in this paper. On the other hand, the results of Walker and Adler show for non-electrolytes and inorganic P that the membrane is capable of maintaining concentration gradients. It seems improbable that the gradients exist merely in virtue of the low permeability coefficients of the solutes concerned, so that changes in the blood concentration are reflected only after some time in corresponding changes in the aqueous humour concentration, since the concentration gradients observed in a large number of experiments were always in the same direction, the serum concentration being in all cases higher than that of the aqueous humour.

As a main conclusion then it may be taken that the aqueous humour is a dialysate from the blood plasma, showing the ionic distribution characteristic of such a system; superimposed on this simple system is the phenomenon of selective absorption which manifests itself in concentration gradients of certain molecules, but most probably not of Na^+ , K^+ and Cl^- .

The relation of these findings to the problem of glaucoma may be briefly indicated. So long as it was maintained that the aqueous humour was a secretory product of "ciliary glands", the normal intra-ocular pressure could be described as the secretion pressure of the glands and its pathological increase in glaucoma could be explained as the result of hypersecretion. By regarding the aqueous humour as a dialysate from blood plasma, the intra-ocular pressure has to be considered as similar to any other tissue pressure, being made up of the hydrostatic capillary pressure partly balanced by the difference in osmotic pressure between the aqueous humour and plasma. To account for the raised intra-ocular pressure in glaucoma now, no such simple explanation is available; there is no change in the protein content of the blood, so that a simple change in the colloid osmotic pressure cannot be said to be the cause of glaucoma, as it is claimed to be the cause of renal oedemas. Similarly there is no evidence that glaucoma is due to raised blood pressure. Thus the necessity for a well founded theory of the conditions which determine the formation of the aqueous humour is apparent.

We gratefully acknowledge our indebtedness to Prof. Drummond for his interest and advice and to the Medical Research Council for personal grants to two of us (H. D. and G. H. B.) and for defraying the whole cost of the work.

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CXIII. A COMPARISON OF THE VITAMIN D CONTENTS OF GUERNSEY AND SHORTHORN BUTTER (MILK).

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(Received March 25th, 1936.)

THE well-known differences in the fat percentages and in the relative carotene and vitamin A contents of the milks of Shorthorn and Guernsey cattle have led us to a comparative investigation of the vitamin D contents of these milks.

EXPERIMENTAL.

(1) *Source of milk.*

Five Guernsey cows and five Shorthorn cows were allowed to run together on the same pasture from about the middle of April until the middle of June, 1935. From the first of May (the last cow joined the group on April 30th) until the end of the experiment the cows received in addition to the pasture an average of 9.6 lb. of concentrates, 24 lb. of mangels and 6 lb. of oat and vetch silage per cow daily in the Guernsey group and 5½ lb. of concentrates, 25 lb. of mangels and 6 lb. of silage similarly per cow in the Shorthorn group. Supplementary feeding to this extent is not common during May and June, but the additional foods were given in this case because the pasture was short and heavily stocked. The difference in the intake of concentrates was partly due to the higher milk yield of the Guernseys and partly to the higher fat content of their milk. They received 4 lb. of cubes for each gallon of milk over 15 lb. per cow per day, while the Shorthorns, producing milk poorer in solids, were given 3½ lb. for similar quantities. The concentrates consisted mostly of cubes compounded from maize meal, middlings, rice meal, decorticated groundnut cake meal, maize gluten feed, linseed cake meal, undecorticated cotton cake, sugar cane molasses, with small additions in the case of two Guernseys and one Shorthorn cow of mixtures of slightly different composition.

All the milk of the cows in the two groups was measured and collected each morning and evening over the period from June 11th, a.m. to June 13th, p.m. both dates inclusive. The fat percentage was estimated twice daily for each cow. From the mixed milks of each group, 6 pints were kept from the evening milk and 9 pints from the morning. The collected milk of each group was separated on the morning of the 14th and churnings of the two creams were done on the 18th of June. The butters were melted at 60°, filtered, and the butter fats were stored at -2°.

Table I gives the ages, dates of calving of the cows, the number of lactation, the date when turned out to pasture and the average daily weight and the fat content of the milk yielded during the 3-day collection period.

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Table I. *Ages, dates of calving, number of lactation, dates of turning out to pasture, milk and fat yields of experimental cows.*

Breed	Name	Wt. kg.	Age (years and months)	Days since calving	No. of lacta- tion	Turned out to pasture	Average daily yield June 11th-13th		
							Milk kg.	Fat kg.	Fat %
Guernsey	Fanny 2	447.7	4, 1	117	2	12. iv. 35	16.25	0.735	4.52
	Peace 2	541.1	8, 2	93	6	18. iv. 35	10.77	0.511	4.74
	Rosey 6	449.5	5, 3	79	3	29. iv. 35	16.33	0.807	4.94
	Valven 2	352.0	2, 8	139	1	12. iv. 35	13.61	0.670	4.92
	Venus 1	450.4	7, 2	106	5	12. iv. 35	21.50	0.888	4.13
	Average	448.1	5, 6	107	3	—	15.69	0.722	4.00
Shorthorn	Flora 16	586.0	5, 7	184	3	18. iv. 35	12.36	0.441	3.57
	Flora 21	495.3	4, 7	87	2	29. iv. 35	14.67	0.454	3.09
	Lily 3	526.2	8, 6	82	6	30. iv. 35	11.83	0.372	3.14
	Lottie 7	446.3	4, 0	288	1	18. iv. 35	13.08	0.455	3.48
	Wistaria 4	551.1	6, 5	163	4	9. iv. 35	14.59	0.568	3.90
	Average	521.0	5, 10	161	3	—	13.31	0.458	3.44

(2) *Biological tests on butter fats.*

The Guernsey and Shorthorn butter fats were tested for their vitamin D content in protective experiments on rats. The technique of these tests has been previously described [Kon and Henry, 1935]. The butters were tested at one level of intake, 0.3 g. of butter fat per rat per day (with the exception of Sundays), on groups of 12 rats and compared with one level of the international standard for vitamin D (V.D. 10) and with negative controls.

The results of the tests are given in Table II.

Table II. *Bone ash % of rats receiving Shorthorn and Guernsey butter fats.*

Negative controls				0.2 Unit Standard				0.3 g. Shorthorn butter fat				0.3 g. Guernsey butter fat			
Rat	Sex	Litter	Ash %	Rat	Sex	Litter	Ash %	Rat	Sex	Litter	Ash %	Rat	Sex	Litter	Ash %
4787	♀	1	29.0	4788	♀	1	42.1	4789	♀	1	39.8	4790	♀	1	44.8
4791	♂	1	24.9	4792	♂	1	45.8	4793	♂	1	38.9	4794	♂	1	36.3
4795	♂	2	29.0	4796	♂	2	47.3	4797	♂	2	38.5	4798	♂	2	38.1
4807	♂	3	27.6	4808	♂	3	43.2	4809	♂	3	38.1	4810	♂	3	40.0
4811	♂	3	24.6	4812	♂	3	46.4	4813	♂	3	36.9	4814	♂	3	36.5
4815	♀	4	25.5	4816	♀	4	44.6	4817	♀	4	33.9	4818	♀	4	39.1
4819	♀	4	29.1	4820	♀	4	39.4	4821	♀	4	32.2	4822	♀	4	35.1
4823	♀	5	26.1	4824	♀	5	48.1	4825	♀	5	45.3	4826	♀	5	39.1
4827	♀	5	27.6	4828	♀	5	45.4	4829	♀	5	33.8	4830	♀	5	42.3
4831	♀	6	32.4	4832	♀	6	46.1	4833	♀	6	42.2	4834	♀	6	48.3
4835	♀	6	29.2	4836	♀	6	48.0	4837	♀	6	43.3	4838	♀	6	46.2
4839	♀	7	30.2	4840	♀	7	45.0	4841	♀	7	42.5	4842	♀	7	39.6
Average			27.93				45.12				38.78				40.45
S.E.M.			±0.67				±0.73				±1.18				±1.19

Guernsey butter has produced a slightly better calcification than the same level of Shorthorn butter, the difference being 1.67 percentage units. This difference is, however, not statistically significant, as its standard error of the mean (calculated from differences between litter-mate pairs on both treatments) is 1.24 and the probability of it being due to chance accidents of sampling 1/5 according to "Student's" *t* test [Fisher, 1932].

The gains in percentage of ash over the negative controls were as follows:

Substance tested	Gain	S.E.M.*
0.3 g. level of Guernsey butter fat	+12.52	±0.93
0.3 g. level of Shorthorn butter fat	+10.65	±1.18
0.2 unit of International Standard	+17.19	±1.06

* In order to take advantage of litter-mate comparisons, the standard errors of the mean are calculated from means of individual differences within pairs and not from differences of means.

From these gains the vitamin D content of the butter fats has been calculated in International Units by means of the equation

$$\text{Gain in ash \%} = 26.33 + 16.6 \log \text{dose (I.U.)}.$$

The method of calculation has been fully described in an earlier paper [Kon and Henry, 1935].

The following values were obtained:

Guernsey butter fat 0.35 I.U. per g.¹

Shorthorn butter fat 0.28 I.U. per g.

They are lower than those usually obtained by us for summer butters [Kon and Henry, 1935].

As already pointed out, the difference between these two values is not statistically significant. The following calculations have been carried out on the actual values, but the statistical limitations of this procedure are realised. The average fat yield of the experimental Guernsey cows works out at 0.722 kg. per cow per day and the corresponding figure for Shorthorns is 0.458 kg. (Table I). On the assumption that the total vitamin D activity of the milk is concentrated in the fat, a Guernsey cow secreted daily on the average 253 I.U. and a Shorthorn cow only half this amount, namely, 128 I.U. This comparison is, however, hardly fair, as the Shorthorn cows were more advanced in lactation than the Guernseys by some 8 weeks and were yielding relatively less milk. When the calculations are carried out per kg. of milk the difference between Guernseys and Shorthorns narrows to 16 I.U. against 10, still an appreciable difference. The effect of the stage of lactation on the vitamin content of milk has not been investigated so far beyond the colostral stage and it is difficult to say whether the slight difference between Shorthorn and Guernsey butters (if at all real) was due to such an effect. Whether or not the present findings of a lack of serious difference in the antirachitic activities of Guernsey and Shorthorn butters would be confirmed at other seasons, or under different nutritional conditions, is a matter of conjecture. If they are provisionally accepted as valid, the relative activities of the milks of the two breeds could be simply expressed by the ratio of the fat percentages.

A comparison of the present results with those obtained by Hess *et al.* [1932] when feeding irradiated yeast to cows of various breeds shows an interesting difference. These authors found that on the same intake of antirachitic material the vitamin D concentration in the butter fat was the higher the lower the total yield of fat.

The relationship does not obtain in the present case and it is significant that cows on pasture are likely to obtain the antirachitic factor not only by ingestion but also through the direct action of the sun. The relative contributions from these two sources are now under investigation in our laboratory.

¹ These values express the antirachitic potencies of the butters as measured by the feeding of butter fat to rats. In making use of these figures the reservations made by Kon and Booth [1933; 1934, 1, 2] should be kept in mind.

SUMMARY.

1. The vitamin D contents of Guernsey and Shorthorn butter fats produced on pasture under similar conditions of feeding and management have been compared. The Guernsey butter fat was found to contain 0.35, the Shorthorn 0.28 I.U. of vitamin D per g., but the difference is not statistically significant.

2. It is suggested that if the relationship is generally valid the relative vitamin D activities of the milks of the two breeds could be simply expressed by the ratio of the fat percentages.

We are indebted to Mr J. Mackintosh for access to the cows, to Miss D. V. Dearden for the churning of butters and to Miss M. Chapman for help with the rats.

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CXIV. FURTHER EVIDENCE FOR THE EXISTENCE OF VITAMIN B₄.¹

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THE existence of a water-soluble thermo-labile nutritional factor distinct from vitamin B₁ or B₂ was first described by Reader [1929]. This substance was designated vitamin B₄ [Reader, 1930, 1], indicating a similarity in respect to solubility to other factors in the vitamin B complex. In a series of publications [Reader, 1930, 2; Barnes *et al.*, 1932; Kinnersley *et al.*, 1933] a method of producing vitamin B₄ deficiency in the rat, a method for assay of this factor and a scheme for concentrating the substance were described.

More recently there has appeared expression of doubt of the existence of vitamin B₄ as a separate entity. Interpretations of feeding experiments have resulted in an opinion that vitamin B₄ deficiency is a chronic phase of vitamin B₁ deficiency and may be cured by excessive doses of vitamin B₁ [see Harris, 1935; O'Brien, 1934; Birch *et al.*, 1935].

In opposition to this denial of the reality of vitamin B₄, we wish to express belief in its existence, with a precise function in the metabolism of at least two species of animals.

Keenan *et al.* [1933] demonstrated the necessity of this factor for growth and normal behaviour of the chick in this laboratory. Concentrates of vitamin B₄, prepared according to the method of Barnes *et al.* [1932], prevented the development of paralytic symptoms in the chick, a syndrome which developed in the animal receiving a diet containing adequate amounts of vitamin B₁. In this species the vitamin B₄ deficiency is characterised by a complete loss of coordination, a tendency to lie on the side and turn cartwheels and finally general prostration and death. A degeneration in the cerebellum of the vitamin B₄-deficient chick was found, similar to the encephalomalacia reported by Pappenheimer and Goettsch [1931]. Improvements in technique and further purification of the constituents of the chick ration [Kline *et al.*, 1936] have given us a valuable method for assay and study of vitamin B₄.

For further proof of identity of the antiparalytic factor with the vitamin B₄ of Reader and associates, it was necessary to produce the deficiency in the rat and to cure it with a vitamin B₄ concentrate. We felt that this was not impossible, because symptoms of vitamin B₄ deficiency have been observed in our vitamin B₁ studies in rats fed diets low in both vitamins B₁ and B₄, as well as in experiments on other factors of the vitamin B complex.

¹ Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

A synthetic diet, designed after that used in the chick experiments but more highly purified, was used. Diet 44_{2A} consisted of:

Dextrin	63.5
Purified caseinogen	24.0
Salts I	4.0
Lard	6.0
Autoclaved liver residue	2.0
Vitamin B ₂ concentrate	≅ 2 % liver extract powder
Cod liver oil	1.0

The dextrin was heated for 24 hours at 120°. Caseinogen, prepared from skim milk, was reprecipitated from dilute NH₄OH solution with dilute HCl at p_{H} 4.6, then washed with distilled water. This treatment was repeated three times. The salt mixture used has been described previously [Kline *et al.*, 1936]. A commercial, water-extracted liver residue, autoclaved 10 hours at 120° to destroy any residual vitamin B₄, was used to supply the essential liver growth factor [Kline *et al.*, 1934]. A vitamin B₂ concentrate, prepared according to the method of Elvehjem and Koehn [1935], was added to the diet equivalent to 2 % liver extract powder.

In our first experiments the technique for producing the deficiency described by Reader was employed. Diet 44_{2A} was made available to suckling rats 10 days old. The mother was removed to a separate cage for several hours daily and fed the stock ration. The young were weaned at 23–25 days of age, with a beginning weight of 40–45 g., and placed in individual screen-bottomed cages. At the first indication of polyneuritis, which occurred usually during the fifth week, a vitamin B₁ supplement was given.

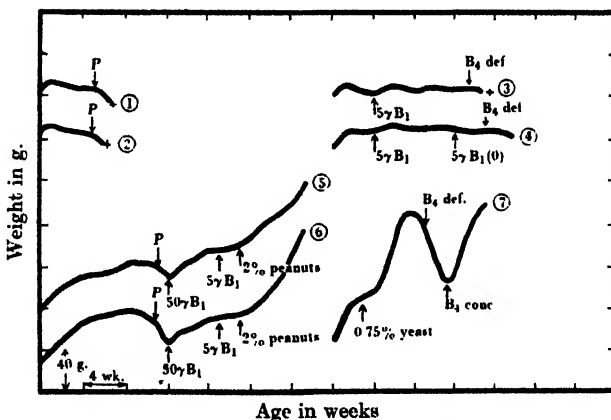


Fig. 1. Growth curves of rats which received basal diet 44_{2A} with the various supplements indicated. *P* denotes the onset of polyneuritis; B₄ def. indicates onset of symptoms of vitamin B₄ deficiency. The vitamin B₁ used was Merck's crystalline B₁ in all cases except curve 4, 5 γ B₁ (O), which was Ohdake's crystalline preparation. The vitamin B₄ concentrate was prepared from peanuts.

The growth curves presented in Fig. 1 are typical records and illustrate the points we wish to bring out. Records of two control animals (curves 1 and 2), which received the basal diet without vitamin B₁ supplement, indicate that polyneuritis was followed by death in the fifth week. Curve 3 demonstrates a

lack of growth response to vitamin B₁ (5 γ daily of Merck's crystalline preparation), with the onset of vitamin B₄ deficiency during the twelfth week, followed by death of the animal. Curve 4 is the record of an animal which received 5 γ daily of Merck's crystalline vitamin B₁ beginning on the 28th day of the experiment, replaced by 5 γ daily of Ohdake's crystalline vitamin B₁ in the twelfth week. Onset of vitamin B₄ deficiency was observed during the fifteenth week. It is apparent that after a very brief rise in weight vitamin B₁ had no further effect on weight increase.

Curves 5 and 6 are records of rats which developed a severe polyneuritis during the eleventh week of experiment. The basal diet was supplemented with 50 γ daily of Merck's crystalline vitamin B₁ beginning at the twelfth week. Polyneuritis was completely cured, but only a slight growth response was obtained in a 5-week period. No change in rate of weight increase was noted when the dose of vitamin B₁ was reduced to 5 γ daily for a 2-week period. Growth was resumed, however, after the nineteenth week of the experiment when 2% of peanuts, as a source of vitamin B₄, were added to the basal diet and the 5 γ daily dose of B₁ continued. In chick assays, peanuts have been found to be an excellent source of this factor. For the rat represented by curve 7, 0.75% brewer's yeast was used as a source of vitamin B₁, added at the beginning of the third week, before polyneuritis had developed. Rapid growth until the eighth week was followed by marked loss in weight with the onset of vitamin B₄ deficiency occurring during the ninth week. Administration of a vitamin B₄ concentrate prepared from peanuts was begun during the eleventh week, allowing an increase in weight of 75 g. in 4 weeks, when the animal was killed for histological study.

Typical symptoms of vitamin B₄ deficiency, similar to those reported by Reader, were observed in these animals. The rats were inactive, sat in an extremely hunched position, and when walking retained the characteristic humped back, "walking high" on the hind legs. As the condition developed there was a noticeable loss of coordination. These animals failed to show the swollen red paws which Reader described.

In a subsequent experiment it was found necessary to use crude liver extract powder (1:20) to furnish vitamin B₂ in place of the vitamin B₂ concentrate

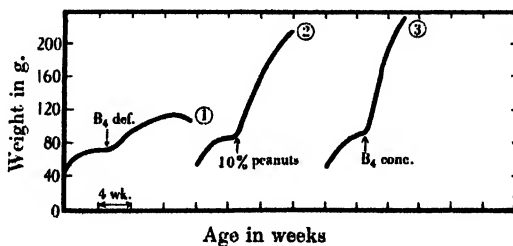


Fig. 2. Growth curves of rats fed basal diet 44_{2H} plus 5 γ daily of vitamin B₁ concentrate.¹ Vitamin B₄ deficiency symptoms were observed at \downarrow . Indicated supplements added at \uparrow .

This diet is designated 44_{2H}. The liver extract powder was aerated in water solution at 100° for 24 hours to decrease the small amount of vitamin B₄ it might contain. The practice of first producing polyneuritis in the rats was found

¹ Vitamin B₁ concentrate was obtained from Sankyo Company, Tokyo, Japan. 1 ml. contains 500 γ of crystalline vitamin B₁, of which 1.5 γ is a rat day dose equivalent to 1 i.u. [Ohdake, 1935].

unnecessary, and from the tenth day the suckling rats had access to diet 44_{2H} containing 0.5 γ of vitamin B₁ per g. of diet. After weaning on the twenty-fifth day the animals received 5 γ of vitamin B₁ daily by dropper.

When a decline in growth rate was noted and symptoms of vitamin B₄ deficiency appeared, supplements were made to diet 44_{2H} *plus* vitamin B₁. These are indicated in Fig. 2. An increase of 25 g. in a 5-week period on the basal diet may be compared with an increase of 139 g. when vitamin B₄ concentrate was used, and 105 g. when 10 % of peanuts was included in the diet. The marked growth increase was accompanied by complete cure of the paralytic symptoms. This response we believe is the result of supplementing the diet with a single factor, vitamin B₄, and bears no relation to vitamin B₁, which at the level of 5 γ daily we have shown to be adequate on a diet low in vitamin B₁. Ohdake [1935] found 1 γ per day to be sufficient for a rat on a diet containing arachis oil which was probably furnishing a considerable amount of vitamin B₄.

DISCUSSION.

If vitamin B₄ is a factor distinct from vitamin B₁ in both constitution and function, it should be unnecessary to produce polyneuritis in the rat prior to the development of vitamin B₄ deficiency symptoms. It is possible that diets which have been used by other workers have not been sufficiently low in vitamin B₄ to limit the intake of this factor to such an extent that symptoms could appear without first lowering the food intake by avitaminosis-B₁. This may explain the apparent vitamin B₄ potency of vitamin B₁ preparations. This relationship has been discussed by Elvehjem and Arnold [1936].

Success in producing vitamin B₄ deficiency in the rat depends to a very large extent upon purification of the various constituents of the diet. We feel that the reprecipitation of the caseinogen is highly essential, and the purification of the dextrin or use of sucrose may aid materially in reducing vitamin B₄ intake. The use of more concentrated supplements furnishing the necessary dietary constituents other than vitamin B₄, as for example the use of crystalline vitamin B₁ in place of yeast and highly potent liver fractions as a source of vitamin B₂, flavin, and other necessary factors, was essential to our success. In continued studies the use of more highly purified concentrates of the B-vitamins will undoubtedly improve our method.

SUMMARY.

Further experimental evidence is offered for the existence of vitamin B₄ and its necessity in the normal nutrition of the rat. Our work confirms the earlier observations of Reader.

The authors are indebted to Prof. S. Ohdake for a generous supply of crystalline vitamin B₁, and to Dr David Klein of Wilson Laboratories for the liver fractions used.

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CXV. *IN VITRO* STUDIES ON LACTIC ACID METABOLISM IN TISSUES FROM POLYNEURITIC CHICKS.¹

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THE idea that disorders observed in polyneuritis are due to the accumulation of intermediate substances originating from carbohydrate breakdown which cannot be metabolised in the absence of vitamin B₁ has prevailed since the pioneer work of Eijkman.

The investigations of Peters and his associates have greatly increased our knowledge of the function of the vitamin and indicate that its action is concerned with the metabolism of lactic and pyruvic acids. Kinnersley and Peters [1929] found an increased amount of lactic acid in the brain of polyneuritic pigeons as compared with the normal. Meikeljohn *et al.* [1932] and Passmore *et al.* [1933] have demonstrated that minced cerebrum from polyneuritic pigeons has a low rate of oxygen uptake in the presence of lactate. This lowered rate of respiration was brought back nearly to normal by the addition of small amounts of vitamin concentrate *in vitro*. However, Meikeljohn [1933] found that brain from polyneuritic pigeons readily removes lactic acid and that the increased oxygen uptake with lactate as substrate was not accompanied by an increased lactate removal.

Birch and Mann [1934] have reinvestigated Boyland's [1933] work on the hypothesis that vitamin B₁ may act as a coenzyme for lactic dehydrogenase. Using a more highly purified dehydrogenase preparation they have shown that the coenzyme for lactic dehydrogenase may be divided into two factors designated by them as coenzyme "A" and coenzyme "B". Evidence was presented to indicate that coenzyme "B" contains a factor responsible for the removal of pyruvic acid. Small amounts of pyruvic acid were shown to exercise an inhibitory effect upon the rate of lactic acid dehydrogenation in this isolated enzyme-coenzyme system. Tests for the vitamin B₁ activity of these coenzymes based upon the cure of bradycardia in polyneuritic rats showed that, although "A" was inactive, "B" exercised some temporary curative effect.

From the work quoted above it is generally concluded that in pigeon brain vitamin B₁ is concerned with some phase of lactic or pyruvic acid metabolism. Since all these tissue oxidation investigations have been carried out upon mature pigeons which have been depleted of their vitamin B₁ stores by polished rice feeding, a slow method requiring at least 30 days to produce head retractions,

¹ Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

we were interested in applying similar methods of study to the polyneuritis produced in chicks by feeding a vitamin B₁-deficient ration developed in this laboratory by Kline *et al.* [1932] and described in detail by Elvehjem [1935]. This ration has the advantage of being so low in vitamin B₁ that polyneuritis is developed in chicks within 2 weeks and yet furnishing ample amounts of the other known factors.

EXPERIMENTAL.

The present investigations have been carried out on young growing White Leghorn chicks weighing 300–500 g. Day-old chicks were placed upon ration 351 [Kline *et al.*, 1934], a complete ration, until they had reached a weight of 250 g. Birds to be used as normals were continued upon this ration, and the experimental chicks were changed to the vitamin B₁-low ration 242 A, which produced acute opisthotonus in 250-g. chicks in 2 weeks. It was found advantageous to grow all chicks to 250 g. weight before placing them upon the vitamin B₁-low ration in order to ensure organs of sufficient size for respiration studies.

Technique employed in respiration studies.

All polyneuritic chicks showing acute opisthotonus and normals were killed by decapitation. After allowing the blood to drain from the neck for 1 min., the cerebrum and other organs to be studied were uncovered and removed to a watch glass where they were gently wiped with filter-paper to remove adhering blood. All tissues were finely minced at room temperature with chromium-plated scissors. Six samples were weighed upon pieces of glass made from coverslips and all introduced at once into the flasks of the Barcroft apparatus,¹ previously filled with buffer, substrate and vitamin (when used) to make a final volume of 3 ml. Rolls of filter-paper were inserted in the absorption tubes which contained 0.5 ml. of 6% NaOH. After the clumps of minced tissue had been broken up with a glass rod, the flasks were attached to the manometers, immersed in the water-bath at 37° and shaken for 10 min. to ensure temperature equilibrium. The stopcocks were closed after the zero reading had been taken, and successive readings were made at 20-min. intervals.

The Ringer phosphate buffer used was made as follows: 20 vols. of *M*/2 Na₂HPO₄ and 80 vols. of Ringer's solution (NaCl 0.9%, KCl 0.025%, CaCl₂ 0.02%, NaHCO₃ 0.015%) were mixed. Enough *N*/10 HCl was added to bring the final solution to *p*_H 7.3. This solution was diluted to 300 vols. and was then *M*/30 in phosphate concentration.

In many experiments Ringer phosphate *plus* pyrophosphate buffer was used and was made as follows: 20 vols. of *M*/2 Na₂HPO₄, 10 vols. of 0.3 *M* Na₄P₂O₇, 10H₂O and 70 vols. of the above Ringer's solution were mixed, adjusted to *p*_H 7.3 with *N*/10 HCl and diluted to 300 vols., making the final solution *M*/30 in phosphate and *M*/100 in pyrophosphate.

The vitamin B₁ concentrate used was Oryzanin Fortior "Sankyo".² An aqueous solution of the concentrate was made to contain 25γ per ml. A 0.1 ml. aliquot containing 2.5γ was added directly to the flasks just before they were attached to the manometers.

Merck's sodium lactate was adjusted to *p*_H 7.3 and diluted to give 3% lactic acid. 0.2 ml. aliquots were added to the right- and left-hand flasks from which an equal amount of buffer was withheld, making 0.2% lactic acid in the flasks.

¹ The Barcroft apparatus used in these studies has been described by Stare and Elvehjem [1933].

² Obtained from the Takamine Corporation, 132 Front Street, New York City.

EXPERIMENTAL RESULTS.

Oxygen uptake studies.

Table I shows the results from 18 experiments to determine if tissues from polyneuritic chicks show any abnormalities in their utilisation of lactate. Although the oxygen uptake of the avitaminous cerebrum in the absence of

Table I. *Effect of lactate upon the oxygen uptake of normal and polyneuritic cerebrum and heart tissue in Ringer phosphate buffer, p_H 7.3, 37°.*

O ₂ uptake in μ l./g./hr.									
		Period (min.)					Period (min.)		
Exp.		0-40	40-80	80-120	Exp.	0-40	40-80	80-120	
Normal cerebrum.					Avitaminous cerebrum.				
1	No lactate	1210	970	720	7	860	830	600	
	Lactate	1430	1630	1250		1310	1140	930	
2	No lactate	1090	930	810	8	980	880	760	
	Lactate	1380	1230	1060		1400	1510	1030	
3	No lactate	980	730	610	9	650	720	460	
	Lactate	1530	1340	1040		1030	1030	850	
4	No lactate	1040	850	710	10	940	870	670	
	Lactate	1010	1370	1210		1310	1190	1030	
5	No lactate	1140	850	620	11	1150	960	720	
	Lactate	1670	1380	1320		1030	1400	1240	
6	No lactate	1140	750	590	12	940	870	440	
	Lactate	1540	1320	1370		1350	1210	970	
Av.	No lactate	1160	850	680		920	860	610	
Av.	Lactate	1430	1380	1210		1260	1270	1000	
Av.	Increase	270	530	530		340	410	390	
Normal heart.					Avitaminous heart.				
13	No lactate	414	330	283	16	193	198	119	
	Lactate	519	388	330		250	190	129	
14	No lactate	220	175	127	17	276	225	298	
	Lactate	365	300	235		279	181	255	
15	No lactate	377	170	167	18	273	195	168	
	Lactate	453	278	258		243	208	184	
Av.	No lactate	337	225	192		247	206	195	
Av.	Lactate	446	322	274		257	193	189	
Av.	Increase	109	97	82		10	- 13	- 6	

substrate was consistently lower than that of normal tissue, the increase in oxygen uptake produced by the addition of lactate to avitaminous cerebrum was not significantly less than that produced by similar additions to normal tissue. Avitaminous heart muscle, on the other hand, seemed to show a marked abnormality in its utilisation of lactate in a majority of cases.

In order to determine whether this apparently decreased oxygen uptake was due to a deficiency of the vitamin in the heart muscle, experiments were carried out with additions of vitamin B₁ concentrate to avitaminous heart muscle respiring in lactate substrate. Similar additions of vitamin B₁ were made to avitaminous cerebrum for comparison with results obtained in Peters's laboratory. Typical results are given in Table II. In avitaminous chick cerebrum the addition of 2.5 γ of vitamin B₁ concentrate had no stimulating effect upon the oxygen uptake in lactate-Ringer phosphate *plus* pyrophosphate solution. However, in heart muscle the vitamin significantly increased the oxygen uptake in the

Table II. *The effect of vitamin B₁ concentrate upon the oxygen uptake of avitaminous tissues.*

LRP = lactate-Ringer-phosphate.

PP = pyrophosphate.

RP = Ringer-phosphate.

V = vitamin 2.5γ.

		O ₂ uptake in μ l./g./hr.				% increase with vit. B ₁
		Period (min.)			Average	
Exp.		0-40	40-80	80-120		
Cerebrum:						
19	RP	940	868	441	750	+ 1
	LRP	1347	1203	959	1170	
	LRP + PP	1321	1128	1080	1176	
	LRP + PP + V	1239	1217	1140	1198	
20	LRP	1233	1342	1200	1258	- 14
	LRP + PP	1287	1240	1155	1227	
	LRP + PP + V	965	1065	1108	1046	
21	LRP	1465	1126	1066	1219	+ 2
	LRP + PP	1352	1130	1126	1203	
	LRP + PP + V	1236	1242	1188	1222	
Heart muscle:						
22 a	RP	281	241	159	227	
	LRP	382	272	207	287	
	LRP + V	404	247	225	292	
22 b	RP + PP	385	213	146	248	12
	LRP + PP	538	247	200	328	
	LRP + PP + V	617	212	265	365	
23	LRP	419	209	106	261	71
	LRP + PP	293	188	207	229	
	LRP + PP + V	562	324	289	392	
24 a	RP	225	190	120	178	
	LRP	313	238	263	271	
	LRP + V	345	241	225	270	
24 b	RP + PP	268	155	100	174	12
	LRP + PP	337	188	279	268	
	LRP + PP + V	395	243	264	300	
25	RP	488	202	237	309	
	LRP	547	417	440	468	
	LRP + V	574	355	387	438	
26	RP + PP	312	229	249	263	20
	LRP + PP	349	382	302	344	
	LRP + PP + V	433	377	432	414	

presence of pyrophosphate. In Exps. 22 b, 23, 24 b and 26 increases of 12, 71, 12 and 20 % respectively were observed in flasks containing added vitamin. In the absence of pyrophosphate (Exps. 22 a, 24 a and 25) the vitamin appeared to have no significant effect upon the rate of respiration of avitaminous heart.

Lactic acid removal by normal and avitaminous cerebrum and heart tissues.

The ability of avitaminous tissues (especially heart) to utilise lactic acid as substrate for oxygen uptake was so much below normal that it was decided to check this lowered rate of respiration by chemical determination of lactic acid removal.

The technique adopted was similar to that employed by Meikeljohn [1933]. After the minced tissues had respired for 2 hours the respirometers were removed

from the bath, and the contents of the flasks immediately washed into small mortars containing 1 ml. of 20% CCl_3COOH (addition of CCl_3COOH to the flasks before removal of the tissues was avoided since it caused particles of tissue to stick firmly to the walls of the flasks). After thoroughly grinding, the contents of the mortars were washed into 15 ml. centrifuge-tubes and centrifuged. The clear liquid was poured into 25 ml. volumetric flasks and the tissue residue extracted twice more with 5 ml. portions of 4% CCl_3COOH . The extracts were added to the 25 ml. volumetric flask in each case and finally brought to 25 ml. vol. Aliquots were taken for lactic acid determinations according to the method of Friedemann and Graesser [1933]. The values reported represent mg. lactic acid removed in 2 hours and 15 min. since the tissues were respiring in the presence of their substrates for 10 min. before the stopcocks were closed and an additional 5-min. period elapsed between the final reading of the manometers and the emptying of the flask contents into the mortars.

Reference to Table III shows that avitaminous cerebrum was able to remove lactic acid at approximately the same rate as normal. However, the rate of removal in avitaminous heart was less than half that of normal tissue.

Table III. *Aerobic removal of lactic acid by normal and avitaminous tissues.*

Fresh wt. of tissue g.	Lactic acid added mg.	Lactic acid present after 2½ hrs. mg.	Lactic acid removed in 2½ hrs.		Fresh wt. of tissue g.	Lactic acid added mg.	Lactic acid present after 2½ hrs. mg.	Lactic acid removed in 2½ hrs.	
		mg.	mg./g.	mg.			mg./g.		
Normal cerebrum.					Avitaminous cerebrum.				
0.130	0	0.41	—	—	0.144	0	0.48	—	—
0.134	5.77	5.43	0.75	5.55	0.142	5.61	5.43	0.66	4.58
0.122	0	0.26	—	—	0.145	0	0.37	—	—
0.124	5.80	5.74	0.32	2.60	0.147	5.61	5.54	0.44	3.00
0.139	0	0.19	—	—	0.205	0	0.38	—	—
0.138	5.39	5.10	0.48	3.48	0.207	5.60	5.41	0.47	2.30
Av. removal			0.52	3.88				0.52	3.30
Normal heart.					Avitaminous heart.				
0.250	0	0.80	—	—	0.256	0	1.15	—	—
0.248	5.77	5.90	0.67	2.70	0.254	5.61	6.37	0.39	1.54
0.210	0	0.74	—	—	0.212	0	0.90	—	—
0.215	5.80	5.97	0.58	2.70	0.209	5.61	6.30	0.21	1.00
0.192	0	0.62	—	—	0.220	0	0.71	—	—
0.188	5.39	5.50	0.51	2.72	0.222	5.60	6.05	0.26	1.17
Av. removal			0.59	2.71				0.29	1.27

A possible fallacy in our calculations requires discussion at this time. It was assumed that the amount of lactic acid which accumulated in the control tissues, to which no additions of lactic acid were made, also accumulated in the flasks containing tissues with added lactic acid. Since this lactic acid accumulates as a result of the action of the glycolytic enzyme system which is dependent upon the oxidation-reduction potential of the suspension [Michaelis and Runnström, 1934], this assumption seems justified. It is believed that the addition of sodium lactate has not inhibited the formation of lactic acid.

Lactic acid dehydrogenase activity of avitaminous and normal tissues.

Lactic acid dehydrogenase activity was measured directly in avitaminous and normal cerebrum and heart tissues by means of the Thunberg [1920] technique. The tissues to be studied were removed from the chick, weighed and

ground with fine quartz sand in 5 cm. mortars. The ground tissues were then washed into tall 50 ml. graduated cylinders with Ringer phosphate solution and diluted with Ringer phosphate to a volume which would make a 5% suspension of the tissue. The contents of the cylinder were thoroughly mixed by shaking, and allowed to settle for 1 min., and 2 ml. aliquots were transferred to Thunberg tubes from the clear tissue suspensions. When substrate was used 2 ml. of 0.5% lactate adjusted to p_H 7.3 were added to the tubes. Ringer phosphate replaced the lactate in experiments where no substrate was used. Exactly 1 ml. of 1:5000 methylene blue was pipetted into the bulb of the Thunberg tube, making the total volume 5 ml. The tubes were evacuated, their contents mixed and placed in a water-bath at 37°. Typical results are given in Table IV.

Table IV. *Decoloration of methylene blue by 5% suspensions of normal and avitaminous tissues in Ringer phosphate and lactate-Ringer phosphate.*

Experiments run at 37°, p_H 7.3 in Thunberg tubes. Values are reported in min. for 80% decoloration of 1 ml. 1:5000 methylene blue.

Normal		Avitaminous		Normal		Avitaminous	
No lactate	Lactate	No lactate	Lactate	No lactate	Lactate	No lactate	Lactate
Cerebrum.				Cerebellum.			
30	7	25	6	24	6	25	6
32	8	25	7	29	8	35	7
29	6	35	7	20	7	30	7
Av. 31 (10)*	7 (11)	29 (6)	7 (6)	25 (8)	7 (10)	29 (6)	7 (6)
Optic lobes.				Heart.			
30	8	25	8	30	4	90	18
32	8	30	8	20	6	60	16
31	10	35	9	40	5	60	24
				13	9	90	60
				30	9	35	10
				60	4	30	12
				11	4	60	30
Av. 31 (8)	9 (10)	31 (6)	9 (6)	29 (7)	6 (7)	61 (7)	24 (7)

* Numbers in parentheses indicate the number of experimental animals used.

The ability of chick cerebrum, cerebellum and optic lobes to reduce methylene blue was not decreased in avitaminosis. However, in avitaminous heart muscle, the results indicated that there was a lowered ability to reduce methylene blue. This subnormal reducing rate still persisted in the presence of lactate. It was found that normal heart tissue reduced methylene blue in one-fourth the time required for avitaminous tissue to reduce the same amount of methylene blue.

In order to determine if this lowered reducing activity in avitaminous heart muscle were a primary result of vitamin B₁ deficiency, additions of vitamin B₁ were made to avitaminous tissue in Thunberg tubes. Ringer phosphate-pyrophosphate buffer was used throughout. It may be seen from the results given in Table V that the *in vitro* addition of vitamin B₁ had no effect upon the reaction. The time required for avitaminous heart to reduce methylene blue was not decreased by vitamin B₁. In order to make the conditions more nearly comparable with those in oxygen uptake studies, experiments were carried out using minced instead of ground heart muscle. But with tissue prepared in this way, as with ground tissue, the vitamin did not affect the rate of decoloration of methylene blue.

Table V. *The effect of vitamin B₁ concentrate upon the rate of decoloration of methylene blue in lactate substrate by avitaminous heart tissue.*

Experiments run at 37°, p_H 7.3 in Thunberg tubes with Ringer phosphate-pyrophosphate buffer. Values are reported in min. for 80% decoloration of 1 ml. 1 : 5000 methylene blue.

Concentration of tissue suspension %	No substrate	Lactate	Lactate + vitamin (2.5γ)
5	40	14	15
5	40	14	15
5	70	16	16
2.5	—	50	50
2.5	—	75	75
2	—	120	120
*	—	75	75
*	250	120	120

* Each tube contained 0.05 g. of scissor-minced heart muscle.

Inhibition by pyruvic acid.

Birch and Mann [1934] showed that small amounts of pyruvic acid inhibited lactic acid dehydrogenation by their purified enzyme-coenzyme system. We have found that pyruvate additions to heart and kidney tissues likewise caused an inhibition of lactic acid dehydrogenase activity, which is especially marked in avitaminous tissue. Typical results are shown in Table VI. In avitaminous tissue

Table VI. *Effect of additions of pyruvic acid upon the lactic dehydrogenase activity of normal and avitaminous tissues.*

Experiments run at 37°, p_H 7.3 in Thunberg tubes. Each tube contained 2 ml. of a 5% suspension of tissue in Ringer phosphate-pyrophosphate buffer, 2 ml. of substrate and 1 ml. of 1 : 5000 methylene blue.

Substrate	Min. for 80% decoloration of methylene blue	Substrate	Min. for 80% decoloration of methylene blue
Normal heart.		Avitaminous heart.	
Nil	65	Nil	68
Lactate	8	Lactate	30
Lactate + pyruvate (2 mg.)	17	Lactate + pyruvate (2 mg.)	70
Lactate + pyruvate (4 mg.)	35	Lactate + pyruvate (4 mg.)	80
Lactate + pyruvate (8 mg.)	40	Lactate + pyruvate (8 mg.)	100
Normal kidney.		Avitaminous kidney.	
Nil	16	Nil	35
Lactate	6	Lactate	20
Lactate + pyruvate (2 mg.)	9	Lactate + pyruvate (2 mg.)	35
Lactate + pyruvate (4 mg.)	11	Lactate + pyruvate (4 mg.)	45
Lactate + pyruvate (8 mg.)	11	Lactate + pyruvate (8 mg.)	50

2 mg. of pyruvic acid were sufficient completely to inhibit lactic dehydrogenase activity. Normal tissue was able to tolerate higher concentrations of pyruvate without complete inhibition of this reaction.

DISCUSSION.

These results are not entirely in agreement with those of Peters and co-workers, who have found that disturbances in carbohydrate metabolism occur principally in brain tissue in polyneuritic pigeons. Rydin [1935, 1] has shown

that the effect of the vitamin upon oxygen uptake of pigeon brain tissue is less marked in pigeons showing only leg weakness than in birds exhibiting acute opisthotonic symptoms. He [1935, 2] has more recently found that the decreased oxygen uptake in skeletal muscle, liver, kidneys and erythrocytes of pigeons fed with polished rice until opisthotonus developed was due to inanition. It must be remembered that the polyneuritis developed in pigeons by a diet composed exclusively of polished rice is a slowly developing deficiency and may be complicated by the absence of other essential factors of the B-complex besides B_1 .

The nature of the polyneuritis which develops in experimental animals upon a vitamin B_1 -low diet appears to be dependent upon the amount of vitamin previously stored and upon the rate at which these stores are depleted. Birch and Harris [1934] suggest from their studies on bradycardia in polyneuritis that rats given no vitamin B_1 have a very low heart rate and no polyneuritic convulsions, while slight traces of vitamin B_1 act by keeping the heart rate at a high enough level to permit the more slowly developing chronic symptoms to appear. Our results with growing chicks with a very high vitamin requirement indicate that with a more rapidly developing polyneuritis, disturbances in tissue metabolism may appear in heart and possibly other organs before the noticeable effects upon the central nervous system are produced.

Although the oxygen uptake of cerebral tissue from polyneuritic chicks is lower than normal, the oxidation of lactate (as measured by the increase in oxygen uptake produced by the addition of lactate substrate and actual removal of added lactic acid) is not influenced by the avitaminosis nor does the addition *in vitro* of vitamin B_1 have a stimulating effect upon oxygen uptake. No diminution in the rates of reduction of methylene blue by cerebrum, cerebellum and optic lobes in the presence of lactate was observed in polyneuritis. However, with avitaminous heart tissue a lowered utilisation of lactate both as substrate for oxygen uptake and for methylene blue reduction was observed. A lowered rate of lactic acid removal was found in avitaminosis. The subnormal rate of respiration could be improved by *in vitro* addition of vitamin B_1 ; however, measurements of the rate of reduction of methylene blue suggest that the action of the vitamin is not primarily associated with dehydrogenation of lactic acid. It may be, as was suggested by Birch and Mann [1934], that the dehydrogenation of lactic acid is inhibited by the accumulation of its own dehydrogenation products and that an increased rate of oxygen uptake resulting from the addition of vitamin B_1 to avitaminous tissue respiring in lactate may be due to the action of the vitamin in the removal of some intermediate in lactic acid oxidation. Unpublished results from this laboratory indicate that the vitamin is closely associated with pyruvic acid metabolism.

SUMMARY.

1. Lactic acid oxidation by normal and polyneuritic chick tissues has been investigated by means of oxygen uptake studies, determinations of lactic acid removal and estimations of methylene blue reduction time. No significant disturbances in lactate oxidation were observed in brain tissues, but in avitaminous heart a decreased rate of oxygen uptake in lactic acid and a decreased rate of lactic acid removal were found.

2. Lactate oxidation is not affected in avitaminous brain by the addition of vitamin B_1 , but in avitaminous heart similar additions produce increased oxygen uptake.

3. Additions of pyruvate inhibited lactic acid dehydrogenase activity to a greater extent in avitaminous heart and kidney than in normal tissues.

4. The action of vitamin B₁ in lactate metabolism is not primarily concerned with dehydrogenation of lactic acid but may indirectly influence the reaction through its participation in some closely associated reaction.

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CXVI. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

VI. THE EXCRETION OF ETHEREAL SULPHATE BY THE RABBIT FOLLOWING THE ADMINISTRATION OF PHENYLGLUCOSIDES.

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FROM time to time it has been suggested that in the dog (and presumably the arguments apply to other animals) glucuronic acid for detoxication processes may be derived from body protein rather than from glucose directly [Thierfelder, 1886; Mandel and Jackson, 1903; Quick, 1926, 1]. Quick [1926, 2] states that "the precursor of glucuronic acid may be derived more readily from glycogen and glycogenetic amino-acids rather than from glucose". Miller *et al.* [1933] suggest that the dog possesses a store of glucuronic acid or can synthesise it from carbohydrates or amino-acids, but that, when this store is depleted, mucin, which contains glucuronic acid, is used as a source for detoxication purposes. In the case of the rabbit Miller and Connor [1933] have shown that this animal is unable to synthesise glucuronic acid from carbohydrates or amino-acids (or at least performs these syntheses very slowly), but that it can utilise glucuronic acid, obtained by digestive processes, when fed with mucin. Miller and Connor therefore suggest that glucuronic acid for detoxication purposes is derived from food sources. That the food material has an influence on the efficiency of another detoxication process has been demonstrated by Abderhalden and Wertheimer [1925] in the cases of the formation of mercapturic and hippuric acids in the rabbit. In the case of glucuronic acid detoxication a somewhat similar influence of diet has been demonstrated recently by Palladin and Persova [1928], by Kusnetzova [1930] and by Makarevitch-Galperin [1930]. From the above data the balance of evidence seems to indicate that the glucuronic acid utilised in detoxication is preformed either in the diet or in the body of the organism. Glucuronic acid itself, when fed alone, is metabolised to a very slight extent and is a comparatively inert substance physiologically [Biberfeld, 1914; Hürthle, 1927; Quick, 1928].

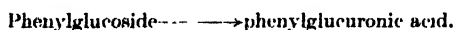
These conclusions are obviously at variance with the Fischer and Piloty [1891] theory of intermediate glucoside formation as a step in glucuronic acid conjugation. Indeed we have already shown [Hemingway *et al.*, 1934] that phenyl- and bornyl- β -glucosides, when perfused through the isolated liver of the dog, are not converted into the corresponding glucuronic acids, although the liver-kidney preparations used were capable of conjugating free phenol and borneol and of excreting these as the glucuronic acids. A repetition of these experiments (hitherto unpublished) using phenyl- α -glucoside likewise gave no glucuronic acid in the perfused liver.¹ None the less experiments have been

¹ This experiment was carried out with the co-operation of Dr A. Hemingway.

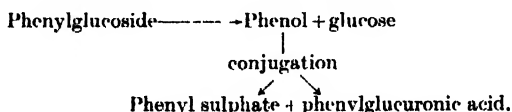
described which have been interpreted as providing evidence in favour of a direct conversion of glucoside into conjugated glucuronic acid. Thus, the experiments of Hildebrandt [1905; 1909] and Hämäläinen [1913] may seem to support this theory, in that conjugated glucuronic acids were excreted after injection and ingestion of certain glucosides. These observations may however be explained on other grounds than direct oxidation of the glucoside to the corresponding glucuronic acid. Thus the glucoside may be hydrolysed *in vivo* to give a free aglucone which may then be detoxicated by conjugation with preformed glucuronic acid. Our own observations lead us to favour the latter view, and references are to be found in the literature which likewise favour this conclusion. Thus Brahm [1899] found that methyl- α -glucoside yielded no glucuronic acid and was excreted unchanged, whilst Schüller's interesting case of phlorhizin has been referred to by Quick [1926, 1]. Schüller [1911] found that on feeding large doses of phlorhizin to rabbits, phlorhizinglucuronic acid was found in the urine. In this conjugated glucuronic acid, however, the original glucose molecule was still intact, and a glucuronic acid molecule had been condensed with one of the free phenolic groups of phloretin, the aglucone of phlorhizin. The experiments now described were designed to elucidate observations of the type made by Hildebrandt.

It is well known that phenol is detoxicated by conjugation with both sulphuric and glucuronic acids. It might therefore be expected that if phenylglucoside were directly oxidised to phenylglucuronic acid no increase in ethereal sulphate excretion would occur. This expectation is strengthened by the observation of Koike and Tosawa [1933] that oral administration of phenylglucuronic acid itself does not give rise to increased excretion of ethereal sulphate in the rabbit. On the other hand if phenylglucoside were first hydrolysed and then detoxicated an increase in ethereal sulphate excretion may be expected, and an observed increase could only be explained by such a hydrolysis.

1st alternative.



2nd alternative.



The present experiments indicate that the second of the above alternatives holds in the case of the rabbit. Two female rabbits were used, one serving as control. In repeat experiments the rôles of control and experimental animal were reversed with the view of eliminating possible individual variations in the two animals. In each experiment the control animal was given a known amount of phenol, whilst the other was given phenyl- β -glucoside sufficient to yield an amount of phenol approximately equal to that given to the control. The phenol and phenylglucoside were fed to the rabbits on the same day, and each animal received food from the same stock, thus eliminating possible dietetic variations which might influence the detoxication process. The results indicate that phenyl- β -glucoside reacts, in regard to detoxication as ethereal sulphate, exactly as if it were free phenol; the percentage of phenol detoxicated and excreted as ethereal sulphate in the glucoside-fed rabbit was the same as that detoxicated and excreted in the control. In both cases very slight traces of free phenol were found in the urine after phenol or glucoside feeding, but the amount of conjugated glucuronic acid excreted, as indicated by the naphthoresorcinol test,

was very little more than normal in each case. The urines after boiling with hydrochloric acid gave a very slight reduction which was probably due to traces of conjugated glucuronic acid rather than to unchanged glucoside.

Other experiments were performed in which phenyl- α - and β -glucosides were injected subcutaneously. There followed an increase in ethereal sulphate output similar to that observed after oral administration, whilst the urine gave very definite indications of the presence of free phenol.

It may therefore be concluded that, as in the case of the isolated liver, the intact animal fails to give any evidence of the direct formation of glucuronides from glucosides administered either orally or subcutaneously. Furthermore phenylglucosides are hydrolysed *in vivo* and, at the levels administered in our experiments, are detoxicated preferentially by conjugation with sulphuric acid, behaving in this respect like the equivalent amount of phenol.

EXPERIMENTAL.

(1) Feeding experiments.

Phenyl- α - and β -glucosides were prepared by the method of Helferich and Schmitz-Hillebrecht [1933] for the synthesis of phenolic glucosides. The final pure crystalline products had physical constants in accord with those already published. The female rabbits used were fed on a mixed diet of bran, oats and cabbage. Urines were collected in a beaker by means of large tinned copper funnels in which the rabbit cages were placed. Etheral sulphate was estimated daily by the gravimetric method. Phenylglucoside and phenol were fed in small gelatin capsules. In the case of phenol, liquid paraffin was added to the capsule to reduce the corrosive action of solid phenol on the stomach. The animals suffered no apparent ill-effects during the entire course of the experiments.

The experimental period lasted 23 days on each of which etheral sulphate was determined. The maximum value found, excluding that of the 1st day after each administration of phenol or glucoside, was for rabbit 11, 27.2 mg.,

Table I.

Rabbit 11, female, 2140 g.			Rabbit 12, female, 1860 g.		Diet, oats and cabbage.		
Controls. Phenol							
Rabbit	Phenol fed mg.	Normal etheral SO ₃ mg.	Etheral SO ₃ on 3rd day mg.	Increase in etheral SO ₃	Phenol conjugated (calc.)	% phenol conjugated with SO ₃	
11	134	15	43	28	32.9	25	
11	195.5	12	82	70	82.2	42	
12	142*	11	30	19.1	22.3	16	
12	142	11	48	37	43.5	31	
Phenyl-β-glucoside							
Rabbit	Glucoside fed mg.	Phenol equivalent mg.	Normal etheral SO ₃ mg.	Etheral SO ₃ on 3rd day mg.	Increase in etheral SO ₃	Phenol con- jugated (calc.)	% phenol con- jugated with SO ₃
12	397	145.5	14.6	41.4	27	31.7	22
12	474	174	11	69	58	68.1	39
11	379	139	12	38	26	30.6	22
11	387	142	14.6	58.5	44	51.7	36

* Some phenol lost during feeding; amount fed uncertain.

for rabbit 12, 23.4 mg. The minimum values were rabbit 11, 6.2 mg., rabbit 12, 6.6 mg. The normal value of the ethereal sulphate output was taken as the average for the 2 days immediately preceding, and for the 2nd and 3rd days following, the administration of phenol or glucoside. The experimentally induced increase was taken as the output observed on the 1st day immediately following the ingestion of phenol or glucoside. The results of the experiments are given in Tables I and II. Fig. 1 records graphically a result obtained for one 5-day period in which rabbit 11 received phenyl- β -glucoside and rabbit 12 phenol.

Table II.

Phenol-fed rabbit		Glucoside-fed rabbit	
Rabbit	% phenol conjugated with SO_3	Rabbit	% phenol conjugated with SO_3
11	25	12	22
11	42	12	39
12	16*	11	22
12	31	11	36

* See Table I.

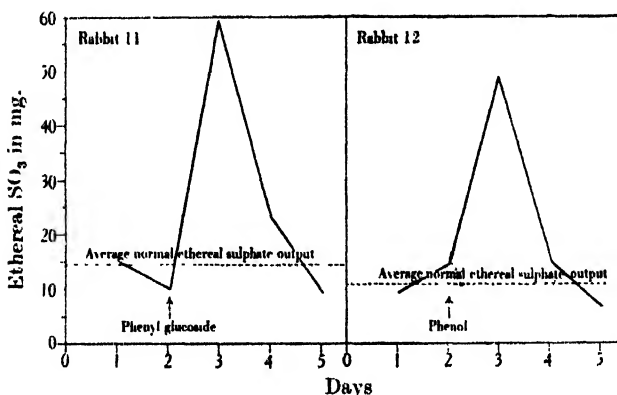


Fig. 1. Increase in ethereal sulphate output following the administration of phenylglucoside contrasted with that following the administration of the equivalent amount of phenol.

(2) Injection experiments.

Phenyl- α - and β -glucosides were also injected subcutaneously and the ethereal sulphate increases were measured as in the experiments already described. Ethereal SO_3 was determined for 25 days. The maximum normal value found was 39.6 mg. and the minimum 7.4 mg. The results are given in Table III.

Table III.

Rabbit C, weight 2740 g.; at the end of experiment, 25 days later, 2690 g.

Glucoside	Normal E.S.	E.S. 3rd day	Increase	% phenol detoxicated
g.	mg.	mg.	mg.	
0.5 β	22.0	78.1	55.2	35.4
0.4 α	22.8	60.2	37.4	30

SUMMARY.

Phenyl- α - and β -glucosides administered orally or subcutaneously to the rabbit are not converted into the corresponding glucuronides but undergo hydrolysis. The liberated phenol is detoxicated and excreted as ethereal sulphate. The phenylglucosides behave in this respect as the equivalent amount of phenol.

The expenses of this work were in part defrayed by a grant from the Medical Research Council. One of us (R. T. W.) is indebted to the Council for a whole-time assistance grant.

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CXVII. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

VII. A NOTE ON THE CONJUGATION OF BORNEOL IN MAN.

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IN two instances reported by Quick [1928] in which borneol was ingested by man, about 80 % was excreted conjugated with glucuronic acid within 10 hours of the administration of 2 g., whilst 69 % was similarly excreted within 6 hours after giving 3·5 g. In the dog Quick has observed 52 % excretion within an unspecified time. We have extended these experiments to a greater number of human cases, and have measured the excretion of conjugated borneol at various times between 6 and 12 hours after ingestion. In agreement with Quick we find that the maximum amount of borneol conjugated with glucuronic acid seems to be about 80 % of that ingested and that, in the normal human being, this limit is reached within 12 hours. Preliminary experiments on certain pathological cases seem to indicate that, where the liver is affected, there may be a marked delay in this conjugation process.

EXPERIMENTAL.

Twenty-four student volunteers of age range 15–25 years, and all free from albuminuria, were each given about 2 g. of borneol accurately weighed in gelatin capsules. All urines collected from the time of ingestion to the end of the experiment (*i.e.* 6–12 hours) were analysed for borneolglucuronic acid. A measured volume of the urine was acidified and extracted for 3 hours in a liquid extractor with ether. The ether was removed and its content of borneolglucuronic acid was hydrolysed with HCl. After neutralising, the glucuronic acid was determined by

Table I.

Subject	Urine vol. ml.	Time hours	% borneol conjugated with glucuronic acid	Subject	Urine vol. ml.	Time hours	% borneol conjugated with glucuronic acid
R. T. W.	910	20·5	78·7	W. G.	310	10	79·3
H. K.	505	12·5	77·5	W. L.	660	10	76·3
G. M. J.	390	12	77·5	W. T.	445	10	75·0
J. H.	345	11·5	78·9	V. M.	310	10	74·8
H. S.	305	11	78·1	J. P. S.	375	(10)?	73·6
A. G.	400	11	76·0	J. H.	630	8·5	70·9
R. G. D.	690	11	74·1	R. T. W.	242	8·5	68·0
R. T. W.	300	10·5	77·9	J. C. J.	360	7	60·5
C. D. C.	432	10·5	77·3	S. W. B.	350	6	58·6
L. P. W.	500	10·5	75·2	K. G. S.	270	6	58·0
L. B.	435	10·5	75·0	M. L.	330	6	57·7
H. M. R.	550	10·5	74·8	H. R.	550	6	57·5
R. T. W.	327	10·5	73·0	C. J. E.	550	6	50·6
J. P.	300	10	81·5				

the Benedict sugar method as modified by Quick [1925] for glucuronic acid.¹ The results are given in Tables I and II. None of the 24 subjects noted any ill-effects following the ingestion of borneol, but in a few cases a transitory dizziness was

Table II. *Individual case.*

Subject	Urine vol. ml.	Time hours	% borneol conjugated with glucuronic acid
R. T. W.	125	4.5	54
"	242	8.5	68
"	327	10.5	73
"	464	13.0	72

mentioned. The borneolglucuronic acid content of each sample of urine was determined as soon as possible after collection. Urines kept for 24 hours even in the refrigerator gave results lower than those obtained for the same urines when fresh.

SUMMARY.

Figures are given, obtained from 24 human subjects, showing the progress of the excretion of bornylglucuronide following the administration of borneol. The maximum amount conjugated and excreted as glucuronide is approximately 80 % of that administered and this maximum is attained in from 10 to 12 hours.

The expenses of this work were in part defrayed by a grant from the Medical Research Council. One of us (R. T. W.) is indebted to the Council for a whole-time assistance grant.

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¹ The Benedict reagent was standardised against hydrated borneolglucuronic acid (0.4163 g. required 11.10 ml. of 0.1052 *N* NaOH; calc. for $C_{16}H_{26}O_7$, $1\frac{1}{2}H_2O$, 11.14 ml.). With this specimen the ratio of the reducing powers of glucuronic acid to glucose was found to be 1.14. Quick [1924] found 1.105 (*cf.* Goebel and Babers [1933] for comparative reducing powers).

CXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLIX. PALITANTIN, $C_{14}H_{22}O_4$, A HITHERTO UNDESCRIBED METABOLIC PRODUCT OF *PENICILLIUM PALITANS* WESTLING.

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(Received March 28th, 1936.)

ALSBERG AND BLACK [1913] described a new mould metabolic product, penicillic acid, $C_8H_{10}O_4$, which they isolated from cultures of *Penicillium puberulum* Bainier. Birkinshaw *et al.* [1936] have recently reported that cultures of Alsberg and Black's strain of *P. puberulum* which, in their hands, gave moderate yields of penicillic acid some seven years ago, no longer produced any penicillic acid in 1933. Search was therefore made for other strains of *P. puberulum*. It was found that Morgan [1933] and Moir [1933] had recorded that this species of fungus is frequently met with in certain discolorations of New Zealand cheese. Through the good offices of the High Commissioner for New Zealand we were able to obtain three different strains of the organism, isolated by Messrs G. F. V. Morgan and J. O. C. Neill from New Zealand Cheddar cheese, and believed by them to be strains of *P. puberulum*.

We found however that when these three strains were grown on Raulin-Thom glucose medium no penicillic acid was formed, but considerable quantities of a hitherto undescribed mould metabolic product were isolated. The question then arose as to the identity or otherwise with *P. puberulum* of the New Zealand cultures. They were therefore submitted to Dr Charles Thom of the United States Bureau of Agriculture. He reported that they were not identical with *P. puberulum*, but were more closely allied to the *P. terrestre* Jensen series, or to *P. solitum* Westling or to *P. palitans* Westling—three species which are difficult to distinguish from each other on purely morphological grounds. Cultures of each of these three organisms, together with the three New Zealand strains and Alsberg and Black's strain of *P. puberulum* were therefore grown under parallel conditions on Raulin-Thom glucose medium. The new metabolic product, for which the name *palitantin* is suggested, was isolated from cultures of the type strain of *P. palitans* [Westling, 1911] distributed by the Centraalbureau voor Schimmelcultures, Baarn, and from each of the three New Zealand strains (none of these four organisms produced any penicillic acid); a different and hitherto undescribed metabolic product, which will form the subject of a future communication, was isolated from *P. terrestre*; no palitantin was produced by *P. puberulum* and nothing of interest could be isolated from *P. solitum*. We are therefore of the opinion that the cultures isolated by Morgan and Neill from New Zealand cheese are more accurately named as strains of *P. palitans* with which they agree closely in morphological characteristics. This opinion is shared by Dr Thom.

Palitantin is a colourless crystalline substance and has the empirical formula $C_{14}H_{22}O_4$. Its molecular constitution has not yet been fully elucidated, but the following points bearing on this question have been established:

1. It is optically inactive, is a neutral substance, and its molecule contains three active hydrogen atoms.

2. It contains an aldehyde group, since it gives a positive Schiff's reaction, and the usual aldehyde derivatives, *e.g.* a mono-oxime, semicarbazone, phenyl- and dinitrophenyl-hydrazone. It is readily oxidised by potassium mercuriiodide to the corresponding monobasic *palitantic acid* $C_{14}H_{22}O_5$.

3. It does not contain a methoxyl group but contains two hydroxyl groups since it gives a dibromobenzoate.

4. It contains at least two double bonds since on catalytic reduction it gives *tetrahydropalitantin*, $C_{14}H_{26}O_4$, which still contains the aldehyde group and is readily oxidised by hypiodite to the corresponding monocarboxylic acid, *tetrahydropalitantic acid* $C_{14}H_{26}O_5$.

5. The function of the fourth oxygen atom has not yet been determined. It does not appear to be present as a carbonyl group since neither a dioxime nor a di-phenylhydrazone (or osazone) could be obtained. Since palitantin only gives a dibromobenzoate it does not appear to contain more than two hydroxyl groups although it contains three active hydrogen atoms.

6. On reduction with sodium amalgam tetrahydropalitantin gives rise to a mixture of two isomeric hexahydro-derivatives, $C_{14}H_{28}O_4$, each of which contains four active hydrogen atoms. Unlike their parent substance, neither of them gives a positive Schiff's reaction. They probably contain an alcohol grouping in place of the aldehyde group originally present.

7. Palitantin, $C_{14}H_{22}O_4$, and tetrahydropalitantin, $C_{14}H_{26}O_4$, are oxidised by Ag_2O to dibasic acids $C_{13}H_{20}O_5$ and $C_{13}H_{24}O_5$ respectively, which were isolated as the dihydrazides.

Further work on the molecular structure of palitantin is in progress and will be reported at a later date.

EXPERIMENTAL.

Cultures.

The following strains of *Penicillium palitans* Westling were used:

(a) L.S.H.T.M. Cat. No. P. 126. Type strain purchased from Centraalbureau voor Schimmelcultures, Baarn, Holland, in August 1931. Received by them from Westling in 1929 and derived from his original strain. (b) L.S.H.T.M. Cat. No. P. 200, New Zealand strain No. 9. (c) L.S.H.T.M. Cat. No. P. 201, New Zealand strain No. 352. (d) L.S.H.T.M. Cat. No. P. 202, New Zealand strain No. 354.

Cultures *b*, *c* and *d* were received by us in January 1934 from Dr T. R. Vernon. They were sent to him in 1933 by Mr J. C. Neill of the Plant Research Station, Palmerston North, New Zealand, who diagnosed them as *P. puberulum*. They were isolated from discoloured New Zealand Cheddar cheese.

Cultural conditions.

The culture medium used throughout this work was a Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; diammonium tartrate, 4 g.; $(NH_4)_2HPO_4$, 0.6 g.; K_2CO_3 , 0.6 g.; $MgCO_3$, 0.4 g.; $(NH_4)_2SO_4$, 0.25 g.; $ZnSO_4 \cdot 7H_2O$, 0.07 g.; $FeSO_4 \cdot 7H_2O$, 0.07 g.; distilled water, 1500 ml. This was distributed in 350 ml. amounts in a number of 1-litre conical flasks, sterilised, sown with a spore suspension, prepared from Czapek-Dox glucose-agar

slopes, of the organism studied and incubated at a chosen temperature until the glucose content, as determined by polarimeter, had fallen below 1 %. The yields of crude palitantin obtained per flask from P. 200, P. 201 and P. 202 were respectively 0.10, 0.125 and 0.16 g. at room temperature (*ca.* 18–20°) and 0.10, 0.10 and 0.02 g. at 24°.

Preparation of palitantin.

Batches of 100 flasks of Raulin-Thom medium were sown with *P. palitans*, L.S.H.T.M. Cat. No. P. 202, New Zealand strain 354. (The strains P. 200 and P. 201 gave smaller yields of palitantin.) The flasks were incubated, originally at the temperature of the laboratory, but in all the later experiments in underground vaults, as the lower temperature prevailing there gave better yields of product. The most favourable temperature was found to be in the region of 10°. When the glucose content, as determined on a sample flask by polarimeter, was reduced to 0.4–0.9 % (usually after 18–22 days at 10°) the metabolism solution, which gave a positive Schiff's reaction, was filtered from the mycelium. Acidification before extraction was found to be unnecessary since palitantin is a neutral compound and undesirable since it gave rise to a product which did not readily crystallise. Extraction of the evaporated metabolism solution also resulted in a sticky product. Hence, in spite of its laborious nature, extraction of the un-evaporated metabolism solution was followed as a routine procedure. The filtrate from each 20 flasks (about 6400 ml.) was extracted with chloroform. Two extractions were performed, each time using 2 litres of chloroform. The chloroform from the second extraction was used for the first extraction of a further batch of 20 flasks. The chloroform was removed under reduced pressure and the extracted material from the whole 100 flasks was combined. The crystals separating from the chloroform were filtered off and washed on the filter with ether, until a pure white product was obtained. A second and usually a third crop was obtained from the mother-liquor after dilution with ether. After complete evaporation of solvent an uncrystallisable syrup always remained, 5–6 g. in weight.

The first crops of palitantin thus obtained were recrystallised from hot water (5 g. to 1 litre) and were then pure enough for most purposes. The second and third crops were twice recrystallised. The average yield at 10° was 10–12 g. per batch of 100 flasks. The experimental details of a number of preparations are given in Table I (batches 1–14).

Isolation of palitantin using the type strain of P. palitans Westling.

One batch (Table I, batch No. 15) of 100 flasks was sown with the type strain of *P. palitans*, L.S.H.T.M. Cat. No. P. 126. The medium, cultural conditions and method of extraction were identical with those described in the previous sections. The CHCl_3 extract (12.13 g.) contained much uncrystallisable syrup as only 3.46 g. of colourless crystalline material were isolated, m.p. 143°. $[\alpha]_{\text{D}}^{25} + 27.2^\circ$. This proved to be a mixture of palitantin with a dextrorotatory acid, which will form the subject of a future communication. The mixture was dissolved in CHCl_3 (200 ml.), water (50 ml.) was added and then, drop by drop, *N* NaOH (4.7 ml.) with vigorous shaking to neutrality to phenolphthalein. The aqueous phase was separated and acidified with HCl. An oil which quickly crystallised separated (1.01 g.) and is the dextrorotatory acid referred to above. The CHCl_3 layer was treated with 4 vols. of light petroleum (b.p. 50–60°). Palitantin, 1.45 g., m.p. 157–9°, optically inactive, separated. Recrystallised from hot water in colourless needles, m.p. 165°, alone or mixed with sample from *P. palitans*, Cat. No. P. 202.

Table I.

Batch	In- cubation period in days	Temperature (°)		Residual glucose by polari- meter %	Final p_H of meta- bolism solution	Titrat- able acidity ml. $N/10$ NaOH per 10 ml.	Yield of crystalline products g.
		Range	Av.				
1. Incubated in laboratory	18	—	—	0.78	2.9	1.42	6.12
2. " "	17	Max. 27	20	0.66	3.1	1.34	3.42
3. Incubated in vaults	18	16-19	18	0.73	3.1	1.26	11.83
4. " "	18	—	—	0.44	3.3	1.20	7.67
5. " "	17	16.5-21.5	19	0.56	3.0	1.52	8.44
6. " "	18	15-21	18	0.44	3.3	1.20	9.67
7. " "	19	10-15	13	0.56	2.9	1.38	13.77
8. " "	19	6-15	11	0.83	2.9	1.67	12.67
9. " "	22	6-13	8	0.64	3.1	1.45	11.13
10. " "	27	8-10	9	0.42	3.4	0.79	13.96
11. " "	22	9-11	10	0.66	2.8	1.12	12.35
12. " "	20	9-11	10	0.85	2.8	1.19	9.81
13. " "	20	10-12	11	0.81	2.9	1.32	10.49
14. " "	26	6-10	8.5	0.70	3.0	1.02	11.00
15. " "	26	5-8	7	0.89	2.8	6.50	3.46

Properties of palitantin.

Palitantin crystallises from water in colourless needles. It may also be crystallised from alcohol, but with more loss. It is only slightly soluble in cold water and ether but is more readily soluble in hot water, alcohol or CHCl_3 . It is optically inactive and is neutral in reaction. It gives a positive Schiff's test and reduces Ag_2O to metallic Ag.

It behaves in a somewhat curious manner in a melting-point determination. At about 135° a film of oily drops forms on the sides of the tube, the solid softens from about 150° and is only completely melted at 163° . The colour is now yellowish. It resolidifies on cooling. This behaviour appears to be characteristic and not due to impurity since it was observed with every sample examined. Moreover, analytical figures agree well with a compound of definite composition, which is also borne out by analysis of derivatives. (Found (Schoeller), sample from P. 202 *ex* alcohol: C, 66.34; H, 8.81 %. Sample from P. 202 *ex* water: C, 66.27; H, 8.70 %. Sample from P. 126 *ex* water: C, 66.12; H, 8.52 %. N, nil; OCH_3 , nil. Mol. wt. (Dr A. E. Oxford) cryoscopic in dioxan 256. $\text{C}_{14}\text{H}_{22}\text{O}_4$ requires C, 66.10; H, 8.73 %. Mol. wt. 254.)

Palitantin contains three active hydrogen atoms since in a Zerewitinoff estimation it afforded 2.7 molecules of CH_4 in anisole at 28° ; 3.1 in anisole at 95° and in pyridine.

Derivatives of palitantin.

Palitantin monosemicarbazone. Semicarbazide hydrochloride (0.11 g.) dissolved in water (2 ml.) was treated with 5 % alcoholic potassium acetate (2 ml.) followed by 0.11 g. of palitantin. A clear solution resulted. After keeping overnight a good crop of crystals separated which were filtered off, washed with water and recrystallised from methyl alcohol. Yield 0.04 g. of colourless needles, m.p. $212-213^\circ$ (decomp.). (Found (Schoeller): C, 58.26; H, 8.00; N, 13.69 %. $\text{C}_{15}\text{H}_{26}\text{O}_4\text{N}_3$ requires C, 57.84; H, 8.10; N, 13.49 %.) The substance is thus a monosemicarbazone of $\text{C}_{14}\text{H}_{22}\text{O}_4$.

Palitantin monophenylhydrazone. Palitantin (0.1 g.) was heated with phenylhydrazine hydrochloride (0.5 g.), anhydrous sodium acetate (0.5 g.) and about 10 ml. of water in a boiling water-bath for half an hour. An orange-coloured

insoluble substance was formed (0.13 g.) which on recrystallisation from alcohol gave colourless needles, m.p. 175–176°, not raised by further recrystallisation. This product proved to be the monophenylhydrazone of palitantin. (Found (Weiler): C, 69.31; H, 8.15; N, 8.61 %. $C_{20}H_{28}O_3N_2$ requires C, 69.71; H, 8.20; N, 8.14 %.) No evidence of a bisphenylhydrazone or osazone was obtained. The orange colour was due to a substance present only in small amount which could not be obtained in crystalline form.

Palitantin mono-oxime and mono-2:4-dinitrophenylhydrazone. Hydroxylamine hydrochloride (0.14 g.) was dissolved in water (2 ml.) and 4 ml. of a 5% solution of potassium acetate in alcohol were added, followed by 0.25 g. of palitantin. No crystals could be obtained so the mixture was evaporated to dryness *in vacuo* and the residue was extracted with ether. On evaporation of the ether an oily product remained which only crystallised after some months. It could not be recrystallised, and melted at 104–106°. It is doubtless the oxime of palitantin. As it was readily soluble in water it was used without further purification to investigate the point as to whether there is a second ketonic or aldehydic group in palitantin. Its aqueous solution was treated with an excess of 2:4-dinitrophenylhydrazine in 2*N* HCl in the hope of obtaining a mixed oxime-2:4-dinitrophenylhydrazone. A yellow precipitate was obtained which was recrystallised twice from methyl alcohol; yellow needles, m.p. 209°. (Found (Schoeller): C, 54.87; H, 6.10; N, 13.35 %. $C_{20}H_{26}O_7N_4$ requires C, 55.27; H, 6.04; N, 12.90 %.) Hence the oxime group has been eliminated and replaced by a dinitrophenylhydrazine group, the product being palitantin 2:4-dinitrophenylhydrazone. There is thus no evidence for another ketonic or aldehydic group in palitantin.

Palitantin di-p-bromobenzoate. Palitantin (0.25 g.) was heated with *p*-bromobenzoyl chloride (0.7 g.) in pyridine (5 ml.) on the water-bath until the mixture began to darken in colour (10 minutes). After cooling, water (40 ml.) was added and the brownish oil which precipitated was separated from the aqueous portion and shaken with a few ml. of sodium bicarbonate solution. After keeping overnight the oil was taken up in a little warm alcohol and water was added until a slight turbidity appeared. The crystals which separated were recrystallised three times in the same manner from aqueous alcohol; colourless plates, m.p. 153–154°. (Found (Schoeller): C, 54.24, 54.33; H, 4.72, 4.66; Br, 26.45 %. $C_{28}H_{28}O_6Br_2$ requires C, 54.18; H, 4.55; Br, 25.77 %.)

Palitantic acid, $C_{14}H_{22}O_5$. Doeuvre's reagent [1927] was found to oxidise the aldehyde group in palitantin to a carboxyl group almost quantitatively. Palitantin (2.54 g. from P. 202) suspended in water (1 litre) was treated with a solution of HgI_2 (4.54 g.) and KI (30 g.) in water (25 ml.). After adding 50 ml. *N* NaOH the mixture was left for some hours with occasional shaking. The solution was filtered through kieselguhr to remove mercury and titrated. It required 20.5 ml. *N* HCl, showing a production of 29.5 ml. of *N* acid in the reaction, the theoretical amount being 30.0 ml. 10 ml. of 2*N* HCl were then added. Palitantic acid began to crystallise immediately. After chilling, the product was collected; long colourless needles, m.p. 146–148°, wt. 1.63 g., which did not require further recrystallisation.

Palitantin from P. 126 behaved in an exactly similar manner with Doeuvre's reagent, giving palitantic acid, m.p. 145–147°, alone or mixed with palitantic acid from P. 202. (Found (Weiler), sample *ex* P. 202: C, 62.06, 62.28; H, 8.11, 8.04 %. Sample *ex* P. 126: C, 62.07; H, 8.25 %. Equiv. by titration, 270.5. $C_{14}H_{22}O_5$ requires C, 62.18; H, 8.21 %. Equiv. titrating as a monobasic acid, 270.)

Tetrahydropalitantin, $C_{14}H_{26}O_4$. Palitantin (1 g.) dissolved in alcohol (100 ml.) was hydrogenated using a Pd-norite catalyst. The absorption of hydrogen was rapid and ceased after 5 min., when the equivalent of 2 mols. of H_2 had been absorbed. The solution still gave Schiff's test. After filtration the solution was evaporated to small volume and about 4 vols. of water were added. The product separated in the form of colourless needles, m.p. 115° , and was filtered off. A further crop was obtained on concentration of the filtrate. The yield was quantitative. The product was recrystallised from alcohol and water (1:5). A sample sublimed in a high vacuum melted at 116° . It solidified on cooling and re-melted at the same temperature. (Found (Schoeller): C, 65.17, 65.20; H, 10.21, 10.29%. $C_{14}H_{26}O_4$ requires C, 65.07; H, 10.15%. Zerewitinoff estimation (Roth): Tetrahydropalitantin affords 3.3 mols. of CH_4 in anisole at 18° , 3.0 in pyridine at 18° .)

Monosemicarbazone of tetrahydropalitantin. Details as for the semicarbazone of palitantin (p. 804). After keeping overnight no crystals had appeared, so water was cautiously added until crystallisation began. The product was recrystallised from a mixture of methyl alcohol and water; colourless rosettes of needles, m.p. $188-189^\circ$ after softening at 186° . There was some darkening at the m.p. (Found (Schoeller): C, 57.22; H, 9.37; N, 13.01%. $C_{15}H_{28}O_4N_3$ requires C, 57.10; H, 9.27; N, 13.32%.)

α -Hexahydropalitantin and β -hexahydropalitantin. Tetrahydropalitantin (0.5 g.) was dissolved in alcohol (50 ml.) and 2.5% sodium amalgam (100 g.) and $N H_2SO_4$ (110 ml.) were added each in ten lots over a period of about 4 hours. The mixture was thus never allowed to become alkaline, since the amount of acid was slightly in excess of that required to neutralise the sodium hydroxide produced. The mixture was heated on the water-bath under reflux. The solution was then extracted with chloroform and the solvent evaporated *in vacuo*. The residue was extracted with boiling ether (about 250 ml.) for some time but a small amount failed to dissolve and was filtered off; wt. 0.04 g., m.p. $140-142^\circ$. When recrystallised from water it separated in the form of colourless plates, m.p. $142-143^\circ$. On chilling the ether filtrate, a further amount (0.08 g.) of the material, m.p. $135-136^\circ$, was obtained. It gave the same product, m.p. $142-143^\circ$, on recrystallisation. It contains 2 atoms of hydrogen more than the original substance, and unlike the latter it gives no Schiff's reaction. It was termed *α -hexahydropalitantin*. (Found (Weiler): C, 64.39; H, 10.87%. $C_{14}H_{28}O_4$ requires C, 64.57; H, 10.84%.) In a Zerewitinoff estimation (Roth) it gave 3.91, 3.93 mols. of CH_4 in pyridine, and 3.34, 3.84 mols. in anisole, at 18° and 95° in each case respectively.

The ether mother-liquor when evaporated to about 10-15 ml. gave 0.10 g. of crystals (plates), m.p. $90-93^\circ$. On recrystallisation from water, then from ether, the m.p. was raised to $98-99^\circ$. There was a syrupy residue from the ether of 0.23 g. The substance, m.p. $98-99^\circ$, proved to be isomeric with *α -hexahydropalitantin* and like that substance it gave no Schiff's reaction. It was termed *β -hexahydropalitantin*. (Found (Schoeller): C, 64.43; H, 10.72%. $C_{14}H_{28}O_4$ requires C, 64.57; H, 10.84%.) In a Zerewitinoff estimation (Roth) it gave 3.83, 3.85 mols. of CH_4 in pyridine, and 3.73, 3.81 mols. in anisole, at 18° and 95° in each case respectively.

Reaction of tetrahydropalitantin with hypoiodite. Tetrahydropalitantin (0.0403 g.) was dissolved in 100 ml. of water by warming. The solution was cooled and to it were added 40 ml. of $N/10$ iodine from a pipette and approximately 50 ml. of $N/10$ NaOH. After keeping overnight 5 ml. of $2N H_2SO_4$ were added and the unused iodine was titrated. A blank experiment was performed on the reagents. The iodine absorbed, 3.07 ml. of $N/10$, corresponded with

1.97 atoms of iodine per mol. of tetrahydropalitantin. The most probable reaction would be an oxidation of an aldehyde group to a carboxyl group and a larger-scale experiment showed that this actually occurs.

Tetrahydropalitantic acid. Tetrahydropalitantin (0.4 g.) was dissolved in 1 litre of water by warming, and to the cooled solution 200 ml. of *N*/10 iodine and 250 ml. of *N*/10 NaOH were added, the latter slowly and with shaking. After keeping overnight 25 ml. of 2*N* H₂SO₄ were added and the solution was titrated with thiosulphate. The iodine used, 2.15 ml. *N*, corresponded with only 69% conversion of aldehyde into acid. The only difference between this experiment and the previous one, except in scale, was a reduction in the excess of alkaline iodine employed. Evidently about 14 times the theoretical amount is required for complete conversion.

The solution was extracted with ether, using about 400 ml. in two lots; the acids were transferred to sodium carbonate solution which was acidified and extracted with ether. The yield of acids, 0.25 g., represents 59% of the theoretical amount. The product set to a mass of crystals. It was recrystallised from ether giving colourless plates, m.p. 110°, to an opaque liquid clearing at 135°. (Found (Schoeller): C, 61.18, 61.12; H, 9.56, 9.48%. Equiv. by titration 275. C₁₄H₂₆O₅ requires C, 61.27; H, 9.56%. Equiv. (titrating as a monobasic acid) 274.) In a Zerewitinoff estimation (Roth) it gave 3.77, 3.92 mols. of CH₄ in pyridine and 1.90, 1.86 mols. in anisole, at 18° and 95° in each case respectively.

Oxidation of palitantin, C₁₄H₂₂O₄, to a dicarboxylic acid, C₁₃H₂₀O₅. Palitantin (1 g.) and silver oxide (5 g.) were mixed in about 150 ml. of water and the mixture was heated in boiling water under reflux for 1 hour. There was reduction of the silver oxide and formation of a silver mirror. The mixture was acidified with 7 ml. of concentrated HCl and extracted with ether. The acids were extracted from the ether by means of aqueous NaHCO₃ and then transferred to ether after acidification. On evaporation of the ether, 0.4 g. of a yellow oily residue remained, smelling somewhat of butyric acid. This was esterified with diazomethane in ether and after removal of the ether and excess diazomethane was treated with 1 ml. of alcohol and 1 ml. of 50% aqueous hydrazine hydrate. In a few minutes crystals appeared, and the mass became semi-solid. The crystals after separation and washing with alcohol and ether melted at 201–202°, after shrinking at 198°, and weighed 0.08 g. The m.p. was unchanged after recrystallisation from alcohol. Analysis showed that this substance was the dihydrazide of a dicarboxylic acid C₁₃H₂₀O₅ (or its lactone C₁₃H₁₈O₄). (Found (Schoeller): C, 54.75; H, 8.50; N, 19.67%. C₁₃H₂₄O₃N₄ requires C, 54.89; H, 8.51; N, 19.72%.)

Oxidation of tetrahydropalitantin, C₁₄H₂₆O₄, to a dicarboxylic acid, C₁₃H₂₄O₅. Details as for the oxidation of palitantin. The syrupy acids weighed 0.49 g. The esters became solid almost immediately on addition of 1 ml. of alcohol and 1 ml. of hydrazine hydrate (50%). The crude product (0.19 g.) after recrystallisation from alcohol yielded 0.13 g. of material melting at 190° after softening at 188°. Analysis showed that it was the dihydrazide of a dicarboxylic acid C₁₃H₂₄O₅ (or its lactone C₁₃H₂₂O₄). (Found (Schoeller): C, 54.23; H, 9.74; N, 19.49%. C₁₃H₂₆O₃N₄ requires C, 54.12; H, 9.79; N, 19.44%.) In an attempt to improve the yield of this product the conditions of the experiment were varied and other oxidising agents, e.g. bromine, CrO₃ in acetic acid and nitric acid, were tried. Bromine did not appear to give rise to acidic products under the conditions employed, and CrO₃ and nitric acid (the latter diluted with water in the proportion 1 in 3) gave rise to the same product as silver oxide in yields of the same order.

SUMMARY.

Palitantin, $C_{14}H_{22}O_4$, a hitherto undescribed mould metabolic product, is formed on Raulin-Thom medium by the type strain of *Penicillium palitans* Westling, and by three different strains of the same species isolated from certain discolorations in New Zealand Cheddar cheese. Various derivatives and reduction and oxidation products of palitantin are described. Palitantin is an unsaturated dihydroxyaldehyde of, at present, unsettled molecular constitution.

We desire to thank Mr W. K. Anslow for much technical assistance in the preparation of quantities of palitantin. We tender our best thanks to the Research Council of Imperial Chemical Industries, Ltd., for a grant to one of us (J. H. B.).

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CXIX. ELECTROMETRIC TITRATION OF INSULIN. PREPARATION AND PROPERTIES OF IODINATED INSULIN.

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THE work to be described in the present paper represents an attempt to obtain accurate data concerning the acid- and base-binding powers of pure insulin by the method of electrometric titration. The experiments have been extended to a study of the physico-chemical and physiological properties of an iodinated product obtained from crystalline insulin by a method which has been used successfully with other proteins [*cf.* Neuberger, 1934, 2] and which appears to be limited in its effect to substitution of the tyrosine groups.

The material used in the investigation was a sample of crystalline insulin kindly given to us by Dr D. A. Scott to whom we wish to express our best thanks.

Existing data bearing on acid- and base-binding powers of insulin.

It is unfortunate that, for the obvious reason of the expense of the material, no complete analysis of the constituent amino-acids of insulin has been made which is sufficiently accurate to allow a close estimation of its acid- and base-binding groups.

Recent determinations of the basic amino-acids of insulin by the Van Slyke distribution method [Jensen and Wintersteiner, 1932, 2; Fisher and Scott, 1934] have led to widely divergent results for the individual bases, although the sums of the total basic amino-acids found by the two groups of workers are not greatly different. Assuming a molecular weight of 35,100 for insulin [Sjögren and Svedberg, 1931] the number of basic groups per molecule (including one terminal α -amino-group) is 30 according to the results of Jensen and Wintersteiner [1932, 2] and 22.5 according to those of Fisher and Scott [1934].

No satisfactory information is available concerning the dicarboxylic amino-acid content of insulin, the only experiments bearing on this point being the qualitative isolation of glutamic acid and failure to obtain aspartic acid from the products of hydrolysis of insulin recorded by Jensen and Wintersteiner [1932, 1]. The value of the isoelectric point of insulin however suggests that the number of free carboxyl groups in the molecule must be at least equal to that of the basic groups.

The figures for the tyrosine content of insulin, *viz.* 12–12.5% [Du Vigneaud *et al.*, 1928; Gerlough and Bates, 1932], rest on a more secure foundation since accurate and specific colorimetric methods are available for the determination of this amino-acid. The value of 12.5% tyrosine, which has been used in the present work, corresponds to 24 tyrosine groups per molecule of insulin; combination of this figure with the approximations deduced above for the basic and carboxyl groups leads to the conclusion that the total base-binding capacity of insulin (tyrosine + carboxyl groups) should be >46 groups per molecule.

Isoelectric point of insulin.

The experiments of Howitt and Prideaux [1932] and those of Wintersteiner and Abramson [1933] suggest that insulin in the amorphous condition or adsorbed on quartz is isoelectric at p_H 5.30–5.35. The optimum p_H for crystallisation of insulin however varies in different buffer solutions from 5.8 to 6.3 as shown by Scott and Fisher [1935]. The latter authors indeed incline to the opinion that the zinc which they find to be an essential constituent of crystalline insulin is bound in salt-like combination with free carboxyl groups.

This view receives some support from the X-ray measurements of Crowfoot [1935] but is rendered of doubtful validity by the following considerations.

(1) Electrophoresis experiments indicate that insulin crystals are isoelectric about p_H 5.0 [Wintersteiner and Abramson, 1933].

(2) The acid-binding capacity of crystalline insulin determined in the present work does not differ greatly from that found for ash-free amorphous insulin by Harvey *et al.* [1934].

(3) If insulin crystals are suspended in CO_2 -free water the solution acquires p_H about 5.3. (The evidential value of this observation is diminished by the very low solubility of insulin at its isoelectric point.)

In the present paper calculations have been made on the assumption that crystalline insulin does not differ in its acid- and base-binding capacities from the pure ash-free protein; the results are therefore subject to future re-calculation if evidence should appear that crystalline insulin is in fact a zinc salt.

Iodination of insulin.

Blatherwick *et al.* [1927] and Jensen *et al.* [1932] have shown that iodine in dilute aqueous solution in the p_H range 6.8–8.0 causes irreversible inactivation of insulin; this inactivation was ascribed by the latter authors to oxidation of disulphide groups. It is possible however to select conditions for the action of iodine on a protein under which the tendency for oxidation is greatly diminished and that for substitution increased.

It was found in the case of zein [Neuberger, 1934, 2] that iodination under such conditions led, so far as could be judged from analytical data, exclusively to the iodination of the tyrosine groups. The same appears to hold good for insulin which takes up an amount of iodine which is almost exactly in agreement with the figure calculated from its tyrosine content.

The titration and the physiological properties of this product, which present points of some interest, are described below.

EXPERIMENTAL.

Titration vessel.

In order to conserve the valuable material it was necessary to devise a method for continuous titration suitable for use with protein solutions having a marked tendency to froth. For this purpose a cell was constructed as shown in Fig. 1 which is self-explanatory.

During the titration purified hydrogen, saturated at the thermostat temperature with the appropriate solvent, was passed through the cell which was rocked in the thermostat with a frequency of about 30 complete rocks per min.; condensation in the connecting tubes was prevented by juxtaposition of a lamp. The rocking device was constructed from Meccano parts and was electrically driven.

The reference electrode was a saturated calomel cell which dipped into a vessel containing saturated KCl (also immersed in the thermostat); junction with the titration vessel was effected by a saturated KCl-agar bridge, the end of which dipped into and moved freely in the saturated KCl vessel.

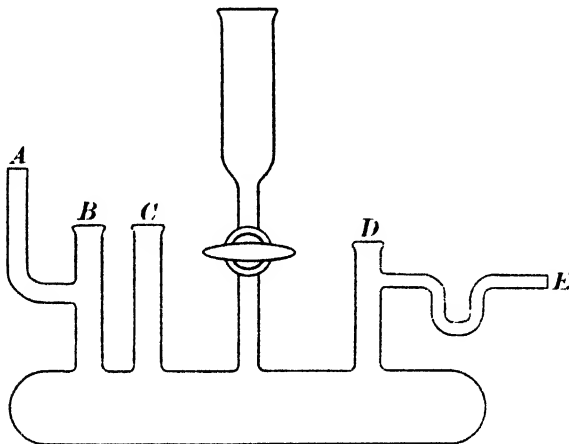


Fig. 1. Titration vessel; $\frac{1}{2}$ actual size. *A*, inlet for hydrogen; *B*, opening for hydrogen electrode, the height of which is adjusted so that the electrode is alternately covered by the solution and exposed; *C*, opening for agar bridge, the end of which is continuously below the surface of the solution; *D*, opening for burette; *E*, outlet for hydrogen.

This size of vessel is suitable for 10–15 ml. of solution.

Titration was carried out at $25^{\circ} \pm 0.05^{\circ}$. The protein was introduced into the cell in the dry state, the solvent being added afterwards. Titrant was added from a microburette in such a manner that the drops fell directly into the solution. Gold-plated platinum electrodes were used and were only introduced into the cell after all the protein had dissolved.

Steady and reproducible potentials were readily obtained with this technique, and no difficulty was experienced from frothing of the solution. Crystalline ovalbumin, when titrated in this apparatus, gave a titration curve in excellent agreement with that determined by Kekwick and Cannan [1936].

Titration of insulin and iodinated insulin.

Over the range p_H 4.3–7.8 insulin is so insoluble in water that it cannot be titrated accurately, whilst in 80% alcohol the zone of low solubility is even more extended. No attempt was made to determine potentials except when the solution was perfectly clear.

Iodinated insulin was titrated on the alkaline side only, since in this case the object was to determine the shift in the curve produced by the alteration in the dissociation constant of the phenolic groups.

Iodination of insulin.

Two preparations of iodinated insulin were made by the following slightly different methods.

(a) Crystalline insulin (200 mg.) was dissolved in a mixture of purified methyl alcohol (10 ml.) and aqueous ammonia (2 ml.; sp. gr. 0.880); the solution was cooled in an ice-salt mixture and treated gradually with iodine in potassium

iodide (2.8*N*) in slight excess over the amount calculated for iodination of the tyrosine contained in the insulin; 0.2 ml. of the iodine solution was used in all. About 45 min. after the last addition of iodine the solution, which was still pale yellow, was centrifuged and the clear supernatant liquid treated in the cold with glacial acetic acid (2 ml.). The clear neutralised solution was diluted with water (about 3 vols.), kept in the cooling mixture for an hour and centrifuged; the p_H of the mother-liquor was 4.5.

The precipitate was warmed to 55° with 15 ml. of 5% acetic acid and the mixture centrifuged; separation was bad but a good flocculation was obtained by adding 5 ml. of 0.1 *N* NaOH to the mother-liquor; the mixture was again centrifuged. (The supernatant fluid, which had p_H 3.2, was adjusted to p_H 5.6; this produced a slight precipitate which was probably a trace of unchanged insulin and was rejected.)

The precipitate was suspended in water and again centrifuged, this process being repeated twice, and was finally dried in a vacuum desiccator; the aqueous washings (p_H 3.9–4.4) were slightly cloudy and a further small flocculation was obtained by adding to them sufficient acetic acid-sodium acetate buffer at p_H 4.5 to make a final concentration of *M*/50; this second precipitate was collected and dried as above.

The total yield was 180 mg. (76% of the theoretical) of a cream-coloured powder. The product gave no Millon reaction but gave on the other hand a strong reaction for the *o*-diiodophenolic grouping with nitrous acid and ammonia. It contained 1.42% H_2O (loss of weight on drying at 120°) and 1.98% of ash; on an ash- and water-free basis the iodine content was 15.4% (calculated on the assumption that the original insulin contained 12.5% of tyrosine: I, 15.4%).

The product differed from insulin in being readily soluble in buffer solutions of p_H 6.8 or over. On the acid side its solubility was profoundly influenced by the presence of salt. Solution was readily obtained below p_H 3.0 in the almost complete absence of salt, but even at p_H 1.5 addition of quite small amounts of sodium chloride caused quantitative precipitation.

Attempts to crystallise the material by methods which have been successfully employed with insulin have failed, but it must be remembered that the amount at our disposal has been so small as to preclude any extended exploration of suitable conditions.

(b) Crystalline insulin (200 mg.) was dissolved in aqueous ammonia (2 ml.; sp. gr. 0.880) and the solution cooled in an ice-salt mixture. Iodination was effected as above with 0.2 ml. of 2.8*N* iodine in potassium iodide. In this case the addition of each drop of iodine solution caused precipitation of nitrogen iodide which rapidly disappeared on stirring. After about half the iodine had been added a white precipitate began to separate and at the end of the addition the mixture had become a stiff jelly. There was no apparent residual excess of iodine.

Addition of water gave a clear solution which was neutralised with glacial acetic acid (2 ml.). The resulting precipitate was collected at the centrifuge (p_H of mother-liquor 5.4) and re-suspended in water; *N* HCl was added to p_H 3.0 when, on warming to 45°, an almost clear solution was obtained; readjustment of the reaction to p_H 4.5 caused sharp flocculation. The precipitate was again collected, and dissolved in water by the addition of *N* HCl to p_H 2.0; the clear solution was treated with a few drops of saturated aqueous NaCl and centrifuged. The supernatant fluid gave no reaction for protein with salicylsulphonic acid; the precipitate was washed at the centrifuge with 0.35% NaCl and dried in a vacuum desiccator.

iodide (2.8*N*) in slight excess over the amount calculated for iodination of the tyrosine contained in the insulin; 0.2 ml. of the iodine solution was used in all. About 45 min. after the last addition of iodine the solution, which was still pale yellow, was centrifuged and the clear supernatant liquid treated in the cold with glacial acetic acid (2 ml.). The clear neutralised solution was diluted with water (about 3 vols.), kept in the cooling mixture for an hour and centrifuged; the p_H of the mother-liquor was 4.5.

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The product differed from insulin in being readily soluble in buffer solutions of p_H 6.8 or over. On the acid side its solubility was profoundly influenced by the presence of salt. Solution was readily obtained below p_H 3.0 in the almost complete absence of salt, but even at p_H 1.5 addition of quite small amounts of sodium chloride caused quantitative precipitation.

Attempts to crystallise the material by methods which have been successfully employed with insulin have failed, but it must be remembered that the amount at our disposal has been so small as to preclude any extended exploration of suitable conditions.

(b) Crystalline insulin (200 mg.) was dissolved in aqueous ammonia (2 ml.; sp. gr. 0.880) and the solution cooled in an ice-salt mixture. Iodination was effected as above with 0.2 ml. of 2.8*N* iodine in potassium iodide. In this case the addition of each drop of iodine solution caused precipitation of nitrogen iodide which rapidly disappeared on stirring. After about half the iodine had been added a white precipitate began to separate and at the end of the addition the mixture had become a stiff jelly. There was no apparent residual excess of iodine.

Addition of water gave a clear solution which was neutralised with glacial acetic acid (2 ml.). The resulting precipitate was collected at the centrifuge (p_H of mother-liquor 5.4) and re-suspended in water; *N* HCl was added to p_H 3.0 when, on warming to 45°, an almost clear solution was obtained; readjustment of the reaction to p_H 4.5 caused sharp flocculation. The precipitate was again collected, and dissolved in water by the addition of *N* HCl to p_H 2.0; the clear solution was treated with a few drops of saturated aqueous NaCl and centrifuged. The supernatant fluid gave no reaction for protein with salicylsulphonic acid; the precipitate was washed at the centrifuge with 0.35% NaCl and dried in a vacuum desiccator.

The yield was 210 mg. (90% of the theoretical) of a product identical in appearance and behaviour with that described under (a) above. As might be expected from its mode of separation this preparation (b) contained higher proportions of water (4.0%) and ash (5.8%); on a water- and ash-free basis however it again contained 15.4% I and had 11.7% N, the latter figure being in good agreement with that calculated from the nitrogen content of the original insulin.

DISCUSSION OF RESULTS.

Acid- and base-binding powers of insulin.

It is not easy to arrive at accurate values for the "maximum" acid- and base-binding capacities of proteins, since most of the latter contain groups which dissociate in very acid or alkaline ranges, under which conditions liquid junction potentials cannot be entirely eliminated. A more serious source of error is the impossibility of making valid blank corrections owing to the fact that the theory of strong electrolytes cannot as yet be applied to multivalent colloidal electrolytes and hence that the activity of hydrogen ions in solutions containing such compounds cannot be evaluated. Furthermore the protein itself may dissociate into smaller molecules at high acidity or alkalinity with resultant liberation of free acidic or basic groups.

Titration in aqueous solution recorded in the present paper have been confined to the range p_H 2.5–11.5 over which the blank correction remains reasonably small and liquid junction potentials can be neglected. The blank correction has been calculated on the assumption that the activities of H^+ and OH^- are equal to their concentrations; this assumption is certainly incorrect and probably leads to values for acid and base bound which are too high; there exists however no sound theoretical basis for any other mode of calculation. For p_{KW} the value of 13.98 has been adopted [Bjerrum and Unmack, 1929].

The results given in Table I and Fig. 2 show that there is no evidence of maximum acid-binding at p_K 2.5. The possibility of including the extreme limits of acid- and base-binding in the titration is greater if the latter be performed in 80% alcohol owing to the effect of the lower dielectric constant in depressing the dissociation of the groups concerned [Neuberger, 1934, 1]; p_H 3.0¹ in 80% alcohol, at which the error introduced by the blank correction is still quite small, corresponds to p_H about 1.5 in water. Examination of this part of the titration curve of insulin in 80% alcoholic solution (Fig. 2) indicates a value of 43 ± 2 groups per molecule as an approximate estimate of the maximum acid-binding capacity of the protein.

Turning to the question of the base-binding power of insulin we find a less satisfactory situation. Owing probably to the fact that for reasons already given no activity correction could be made, the base-binding at p_H 11.5 seems unreasonably high in comparison with the base-binding calculated from analytical data (*v. supra*). Titration in alcoholic solution does not help greatly in the alkaline range. Whereas in the acid range the titration curve in alcoholic solution lies about 1.3 p_H units above that in aqueous solution in accordance with expectation [Neuberger, 1934, 1, 2] in the alkaline range the shift of the alcoholic titration curve varies from a small amount at moderate alkalinity to a large amount at high alkalinity; this deviation is due to the fact that in the range under discussion two sets of groups are dissociating, namely basic groups (*e.g.*

¹ The term p_H in so far as it applies to alcoholic solutions is used in the conventional sense indicated by Neuberger [1934, 1].

Table I. *Titration of insulin.*

A. With acid. 75 mg. insulin; 11 ml. water. Temp. 25°.

ml. 0.1 <i>N</i> HCl	p_H	ml. 0.1 <i>N</i> HCl corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
0.56	3.368	0.510	71.0	25.0
0.60	3.286	0.540	75.0	26.5
0.66	3.159	0.580	81.0	28.4
0.74	3.000	0.623	87.0	30.5
0.82	2.858	0.654	91.5	32.0
0.88	2.750	0.670	93.5	32.9
0.95	2.668	0.694	97.0	34.0
1.00	2.607	0.710	99.3	34.8
1.10	2.506	0.723	101.0	35.5

Reversed titration.

ml. 0.1124 <i>N</i> NaOH	p_H	ml. 0.1 <i>N</i> HCl corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
0.21	2.806	0.622	87.0	30.5
0.39	3.091	0.560	78.3	27.5
0.50	3.362	0.483	67.5	23.7
0.60	3.689	0.400	56.0	19.6
0.65	3.870	0.353	49.3	17.3
0.70	4.059	0.304	42.5	14.9
0.75	4.241	0.250	35.0	12.3

B. With alkali. 75 mg. insulin; 12 ml. water. Temp. 25°.

ml. 0.1124 <i>N</i> NaOH	p_H	ml. 0.1 <i>N</i> NaOH corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
1.5	11.342	1.370	191.5	67.2
1.6	11.392	1.423	199.0	69.8
1.8	11.487	1.570	219.5	77.0

Reversed titration.

ml. 0.1 <i>N</i> HCl	p_H	ml. 0.1 <i>N</i> NaOH corrected	Mol. $\times 10^5$ H ⁺ bound per g. protein	Mol. $\times 10^5$ H ⁺ bound per g. mol. protein
0.2	11.419	1.435	200.6	70.4
0.3	11.351	1.390	194.0	68.2
0.4	11.291	1.330	186.0	65.3
0.5	11.232	1.264	177.0	62.0
0.6	11.167	1.200	168.0	58.9
0.7	11.044	1.152	161.0	56.5
0.8	10.963	1.082	151.0	53.1
0.9	10.826	1.020	143.0	50.0
1.0	10.709	0.940	131.0	46.1
1.1	10.506	0.870	122.0	42.7
1.2	10.267	0.790	111.0	38.8
1.3	9.952	0.710	99.0	34.8
1.4	9.692	0.620	88.0	30.4
1.5	9.173	0.520	73.0	25.5
1.6	8.337	0.420	59.0	20.2
1.7	7.967	0.320	45.0	15.9

guanidino-groups) which are little affected and phenolic groups which are considerably affected by change of solvent.

Between the isoelectric point and p_H 8.5 the base-binding amounts to 21–22 groups per mol. of protein; from p_H 8.5 to 9.5 there is little buffering, as is indeed the case with most proteins. As to the total base-binding between the isoelectric point and p_H 11.5 it is only possible to say that this approximates to 60–70 groups per mol. of protein; from this and from the knowledge of the number of tyrosine groups present it may be calculated that the insulin molecule contains at least

30–35 free carboxyl groups. The protein is indeed remarkable as a whole for the very large number of ionisable groups which it possesses.

It is apparent from Table I and Fig. 2 that reversed titrations gave results in satisfactory agreement with those of the direct titrations in spite of the small change which must have occurred in the ionic strength of the solution; this indicates that no irreversible change was produced in the protein by exposure to high degrees of acidity and alkalinity, a supposition which was confirmed in so far as the aqueous titrations were concerned by the recovery of 75 % of the starting material in crystalline form: the simple recrystallisation of insulin gives

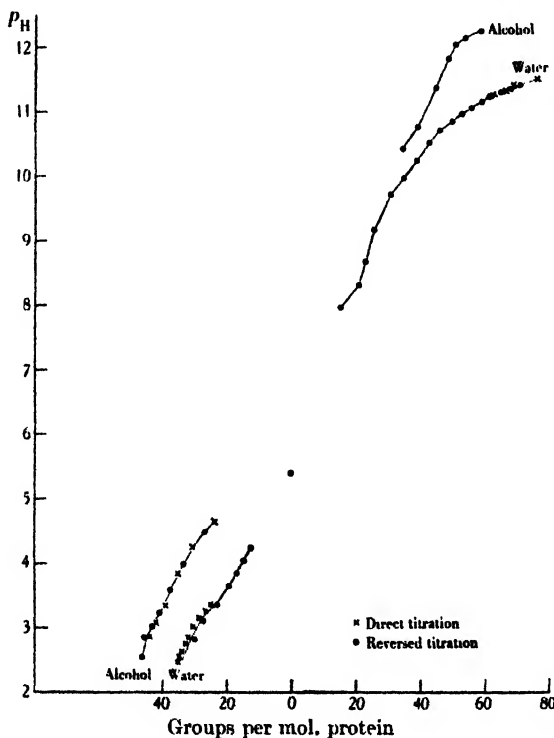


Fig. 2. Insulin.

a yield of the same order [Scott, 1934]. The possibility of reversible dissociation at high alkalinity cannot however be excluded; such dissociation may indeed contribute to the abnormally high figure for the total base-binding.

An electrometric titration of insulin has been reported by Harvey *et al.* [1934]. It is difficult to compare the results of these authors with those obtained in the present communication since an amorphous preparation of insulin was used and no details of the technique of titration are given. Nevertheless the value obtained by them for the acid-binding power of insulin in aqueous solution is in agreement with that determined by us.

Iodinated insulin.

Analyses by earlier workers indicate the presence of 23–24 tyrosine groups in the molecule of insulin; the iodine content of our fully iodinated product corresponds with 24 tyrosine groups per mol. on the assumption that the iodine

has entered exclusively the 3:5-positions of the tyrosine residues. The titration curve of iodinated insulin over the range p_H 6.6–11.5 is shown in Fig. 3 in which is included, for purposes of comparison, the titration curve of insulin over part of the same range. The shift in the titration curve owing to the diminution of the p_K of the phenolic groups in the iodinated product can be clearly seen, as can also the fact that the total base-binding of the protein is unaffected by iodination.

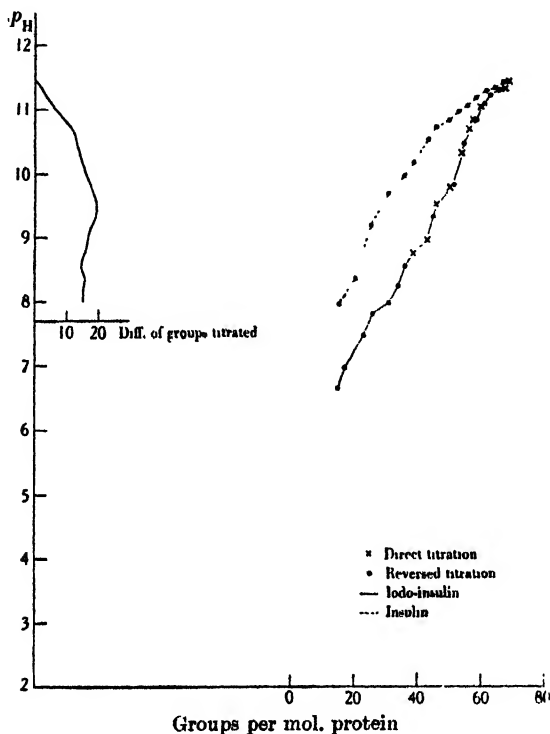


Fig. 3. Iodinated insulin.

The curve on the left of Fig. 3 has been obtained by plotting the difference of the number of groups titrated in insulin and iodinated insulin against p_H . The maximum value of this difference is 20 and is therefore somewhat smaller than that calculated from analytical data (23–24; *v. supra*); this slight discrepancy is partly to be explained by the extended range over which the phenolic groups of insulin dissociate and the consequent overlap of the titration curves of insulin itself and of the iodinated product. Such a discrepancy would also be caused by error in the isoelectric point (p_H 5.5) assumed for iodinated insulin; that no great error of this kind was made is shown by the identity of the total base-binding capacities of iodinated insulin and insulin itself.

The general conclusion that the phenolic groups of insulin are free and that these groups alone are affected by iodination under the conditions employed in this work is supported by the results of the titration; it is further reinforced by the extended range of solubility of iodinated insulin in the neighbourhood of p_H 7.0 which has already been mentioned in the description of its properties and which is shown graphically in Fig. 3.

Effect of iodination on physiological properties of insulin.

Since chemical evidence favours the view that iodination under the conditions which we have employed induces no change in the molecule of insulin other than substitution of the tyrosine residues it became of interest to examine the physiological properties of the iodinated product.

For co-operation in this part of the work we are greatly indebted to Mr H. P. Marks, whose results may be summarised as follows.

Mouse tests. The iodinated product caused no convulsions in mice in doses as high as the equivalent of 1/5 unit of the original crystalline insulin, whether given subcutaneously or intravenously; a dose equivalent to 1/4 unit of the original material injected intravenously caused convulsions in 12/20 mice. This would correspond to an activity about 15% of that of the original insulin.

Rabbit tests. The iodinated insulin had very little hypoglycaemic action in rabbits when given by subcutaneous injection; thus in one case iodinated insulin equivalent to 10 units of original insulin gave less effect than 1/2 unit of the latter. After intravenous injection on the other hand the same dose (10 units) of iodinated insulin lowered the blood sugar to an extent of the same order as the lowering produced by 1 unit of insulin.

The general conclusion to be drawn from these results is that the iodinated product retains 5-10% of the physiological activity of the original insulin.

Removal of iodine from iodinated insulin.

It is evident that whilst the results recorded above leave no doubt that the physiological activity of insulin has been greatly diminished by iodination they leave much to be desired from the point of view of quantitative accuracy, since there is a discrepancy between the effects of the iodinated product when given by the subcutaneous and oral routes, which makes its comparison with insulin difficult. Thus no convulsions were produced in mice with any subcutaneous dose of iodinated insulin although an intravenous dose was effective; this is in direct contrast with the greater efficacy of subcutaneous than intravenous injections of insulin itself. Again in rabbits the activity of iodinated insulin appeared to be about 2.5% of that of insulin by the subcutaneous test and 10% by the intravenous test.

The relatively lower effect of subcutaneously injected iodinated insulin suggests that its absorption may be delayed in comparison with that of insulin. In this connection the possibility was considered that the residual activity of the iodinated product might be due to the persistence of a small amount of unchanged insulin, the absorption of which was delayed by its association with a preponderance of totally inactivated material; such a possibility was however practically excluded by the observation that insulin could exercise its normal effect in mice when administered subcutaneously in admixture with the iodinated material.

A doubt nevertheless remained whether the loss of activity on iodination represented total inactivation of 90% of the insulin, the remainder being unchanged or 90% inactivation of all the insulin. In order to gain further information on this point attempts were made to remove the iodine from the iodinated product in the hope that restoration of physiological activity might be obtained. The experiments about to be described will show that this hope has been partly realised.

It will be apparent that the major difficulty to be faced in these experiments was the search for a method of removing iodine which would not at the same

time cause physiological inactivation through the induction of other changes in the molecule. Insulin is well known to be irreversibly inactivated by treatment with reducing agents even under the mildest condition, such inactivation being accompanied by exposure of sulphhydryl groups. The only method which offered any prospect of success therefore appeared to be that of catalytic reduction which was unlikely to attack the disulphide linkages in the protein.

Catalytic dehalogenation of organic compounds is usually effected in strongly alkaline solution with palladium deposited on calcium carbonate or barium sulphate; in this case the use of strong alkali was excluded for obvious reasons and it was therefore decided to use 50 % aqueous pyridine as solvent, a preliminary experiment having shown that diiodotyrosine could be satisfactorily deiodinated in this medium.

Iodinated insulin therefore (100 mg.) was dissolved in a mixture of equal parts of water and pure redistilled pyridine (10 ml.) and the mixture shaken in an atmosphere of hydrogen in presence of palladium-barium sulphate catalyst (1 g.). Uptake of hydrogen ceased after about 4 hours; the amount absorbed by the catalyst itself was so much greater than that taken up by the protein that the latter quantity could not be determined with any accuracy. The catalyst was filtered off and the solution evaporated under diminished pressure to remove pyridine; adjustment of the reaction to p_H 5.5 caused flocculation of the protein, which was separated on the centrifuge, redissolved in aqueous pyridine and subjected to further hydrogenation with a fresh lot of catalyst. The whole process was then repeated a third time.

The combined mother-liquors (which showed positive reactions for iodide of decreasing intensity) were freed from pyridine by distillation under diminished pressure after addition of potassium hydroxide; the iodide was then determined by oxidation with bromine followed by titration with thiosulphate and was found to correspond to 10.4 mg. of iodine or approximately 70 % of that in the starting material.

The protein was dissolved in water acidulated with hydrochloric acid and reprecipitated at p_H 6.2 from a phosphate buffer (total volume 50 ml.) under the conditions described by Scott [1934] for the crystallisation of insulin. No crystallisation occurred but a flocculent precipitate slowly separated which, after keeping at 0° overnight, was collected and dried. The yield was 35 mg. of a product which gave a strong Millon reaction and only a faint reaction for the *o*-diiodophenolic grouping. It contained 4.2 % of iodine, from which it may be concluded that approximately 2/3 of the phenolic groups had been restored to their original condition. The nitroprusside reaction for sulphhydryl groups was entirely negative.

Since the amount of material available was so small it was decided to subject the partly de-iodinated product to physiological test rather than to attempt more complete removal of iodine.

Fig. 4, which is based on tests kindly carried out for us by Dr H. P. Himsworth, shows in a striking manner the very considerable degree of re-activation which accompanies the removal of iodine. The upper curve represents the effect on the blood-sugar of a rabbit of the intravenous injection of 1/50 mg. of fully iodinated insulin and the lower curve the effect of the same dose of the partly deiodinated product. From the lower curve it may be deduced that the latter product possessed at least 50 % of the activity of crystalline insulin. A test on mice carried out by Mr Marks showed that the deiodinated product produced convulsions in mice when given subcutaneously in the ordinary way and indicated an activity in the neighbourhood of 30 % of that of insulin.

In view of the residual iodine content of the final product an activity of about 60 % of that of crystalline insulin was the maximum to be expected. The results of the rabbit test (Fig. 4) are in accordance with this expectation; those of the mouse test are somewhat lower. It is however clearly established that deiodination has effected a re-activation which is roughly proportional to the amount of iodine removed and therefore to the restoration of the original phenolic groups.

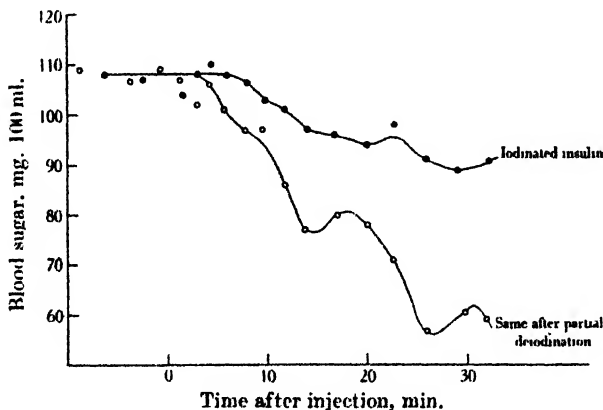


Fig. 4. The curves represent the effects of intravenous injection of 1/50 mg. of the respective products into a rabbit.

It seems reasonable then to conclude that the phenolic groups of insulin are of importance in relation to its physiological activity, since this activity can be almost completely abolished by loading the phenolic groups with iodine and can be restored in approximate proportion as the iodine is removed. We are inclined to believe that the residual activity of the fully iodinated product is intrinsic to this product and is not to be ascribed to the persistence of unchanged insulin although we are unable to offer incontrovertible proof of this point.

It may be pointed out that our conclusion is in accordance with the observations of Jensen and Geiling [1928] and of Freudenberg and Eyer [1932-33] on the partial re-activation of acetylated insulin; the treatment of the acetylated product with dilute alkali adopted by these workers is such as to hydrolyse *O*-acetyl but not *N*-acetyl groups, and the partial reactivation obtained is almost certainly associated with regeneration of free phenolic groups.

SUMMARY.

1. Crystalline insulin has been titrated electrometrically, in both aqueous and 80 % alcoholic solutions, in a specially designed apparatus.
2. From the results it is deduced that insulin has an acid-binding capacity of 43 ± 2 groups per molecule and a base-binding capacity in the neighbourhood of 60-70 groups per molecule.
3. The interpretation of these results in relation to existing analytical data concerning insulin is discussed.
4. Insulin has been iodinated; evidence is presented that the iodinated product differs from insulin only in that the tyrosine groups are substituted with iodine in the 3:5-positions.

5. It is shown that iodinated insulin retains only 5–10 % of the physiological activity of the parent substance, but that partial removal of iodine by catalytic reduction is accompanied by approximately proportional restoration of activity.

One of us (A. N.) wishes to express his gratitude to the Academic Assistance Council for a personal grant during the tenure of which this work was carried out.

We desire to acknowledge our indebtedness to Mr T. H. Mead for his valuable assistance in the construction of the apparatus for continuous titration.

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CXX. THE ANION AND CATION CONTENTS OF NORMAL AND ANAEMIC BLOODS.

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IN the course of investigations on the permeability of erythrocytes, it was noticed that the potassium content and osmotic pressure, as judged from the total ionic concentration, of erythrocytes from cases of acholuric family jaundice, were much diminished, whilst the osmotic pressure of the plasma was about normal. In view of the contradictory nature of these observations, a more complete survey of the several constituents of the cells and plasma in various normal and abnormal conditions was undertaken.

The conditions investigated fall into five groups: normal; microcytic anaemia; macrocytic anaemia; myelosclerosis; acholuric family jaundice (congenital haemolytic icterus).

Potassium, sodium and chloride were determined in cells and plasma; haemoglobin and water were estimated in the cells and bicarbonate in the plasma, whilst cell bicarbonate was calculated from the formula

$$[\text{HCO}_3^-]_{\text{cell}} = \frac{[\text{HCO}_3^-]_{\text{plasma}}}{k} \times \frac{[\text{Cl}^-]_{\text{cells}}}{[\text{Cl}^-]_{\text{plasma}}}.$$

The value of k (which from the Donnan equation should be 1) is between 0.8 and 0.9. It has been given the value 0.85 and the error in cell HCO_3^- calculated in this way is not likely to exceed ± 1 m.eq.

Plasma water was assumed to be 93 %. Deviations from this average figure are almost certainly less than ± 1.5 %.

Further, in order to correlate the findings with erythrocyte destruction and regeneration, the percentage of reticulocytes, the plasma bilirubin and the erythrocyte fragility in solutions of sodium chloride were investigated.

Methods.

Heparinised blood was centrifuged under liquid paraffin at 4000 r.p.m. for 30 min. in tubes drawn out into sealed graduated and calibrated capillaries. Part of the supernatant plasma was removed for analysis and the residue washed away. The volume of cells was measured and the cells analysed. The object of the liquid paraffin was to prevent evaporation of plasma during centrifuging. To some extent it may also check loss of CO_2 , because its viscosity prevents mechanical agitation of the plasma surface.

Sodium was estimated by the method of Oberst [1935]. For plasma, 0.05 ml. was used, the error being ± 2 %. In the case of cells 0.15 ml. was used, the error being not greater than ± 5 % (equivalent to ± 0.5 % of the total base present). Oberst points out that plasma contains about twenty times as much sodium as do cells so that the presence of even a trace of intercellular plasma will give a figure for cell sodium which is relatively much too high. He recommends that the cells be washed with a sodium-free fluid consisting of dialysed plasma whose osmotic pressure is restored by addition of potassium chloride. The use of such

a wash-fluid, if not actually objectionable owing to the possibility of removing sodium from abnormal cells, is time-consuming and unnecessary, for if Oberst's own figures are examined it will be seen that the subtraction of 9.2 mg./100 ml. from the sodium content of unwashed cells gives a very close approximation to the sodium content of the corresponding washed cells. It can in fact be shown that the intercellular plasma is about 2.8% by volume, which would correspond to an apparent increase in cell sodium of 9.2 mg./100 ml. Thus, if 0.1 ml. centrifuged cells is suspended in 2 ml. saline and the suspension re-centrifuged, the protein content of the supernatant saline (consisting of plasma washed out from between the cells) can be estimated and if the protein content of the corresponding plasma be known, the plasma content of the supernatant saline, and hence of the centrifuged cells before suspension, can be determined. The protein content of the supernatant saline was estimated by nesslerisation after oxidation, and also with Folin's phenol reagent, a blank determination being carried out on the supernatant saline after precipitation of the protein with sodium tungstate and sulphuric acid.

In thirteen of Oberst's experiments the sodium content of washed cells, calculated in the above way, differed from the estimated values as follows: +50, +19, +16, +8, +6, -5, -4, +2, +2, -2, -2, -1 and 0%. If the first three results are excluded the differences between the calculated and estimated figures are well within the region of experimental error.

The technique of washing cells, moreover, is objectionable on other grounds; thus whilst it is true that intercellular plasma gives incorrect and weighted results for cell sodium, the same observations apply to other ions and as it is impossible by using different wash-fluids to obtain absolute figures for each of these constituents, it is not reasonable to attempt this in the case of sodium alone. The supernatant plasma above the centrifuged cells is presumably in equilibrium with intercellular plasma and this in turn is in equilibrium with the cells. For practical purposes of comparing and contrasting plasma and cell ion distributions it is essential to consider the supernatant plasma as one phase and to group the cells and intercellular plasma together as the other phase.

Potassium was determined on a protein-free filtrate by the method of Kramer and Tisdall [Maizels, 1935], duplicates on 0.1 ml. cells differed by less than 4% and on 0.6 ml. plasma by less than 10%. These results were 1 or 2% lower than those obtained with ashed cells. For chloride, the method of Claudius [1924] was used on 0.05 ml. plasma and 0.1 ml. cells, and for plasma bicarbonate that of Payne as described by Maizels *et al.* [1930] on 0.1 ml. material. Water was estimated by heating 0.1 ml. cells at 105° to constant weight. The errors of these three determinations were within $\pm 2\%$.

Haemoglobin was estimated by the method of Haldane, the figure 100% corresponding with 138 g. per litre of blood and from this and the haematocrit value the amount of haemoglobin per litre of cells was calculated. The amount is normally about 6 mM with respect to cell water, but as Adair [1928; 1935] has shown, this is osmotically equivalent to an approximately 12 mM solution.

In addition to the chemical estimations, the number of erythrocytes per μ l. of blood was counted and from this and the haematocrit value, the mean cell volume was derived.

There are certain advantages and disadvantages inherent in the methods employed of centrifuging blood in narrow graduated tubes. Intercellular plasma is reduced to a minimum and a very small volume of packed cells can be measured accurately so that it is possible to carry out all the estimations on about 1.5 ml. centrifuged cells instead of on 10 ml. and therefore, in the case of anaemic

patients, it suffices to withdraw only 6 ml. blood instead of 40 ml. On the other hand, loss of CO_2 is not prevented and only partial oxygenation of the haemoglobin of the venous blood may occur: since both these processes affect the distribution of water and electrolytes between cells and plasma the latter cannot be investigated with the accuracy achieved by Van Slyke and Henderson. However, the method permits certain conclusions to be drawn and it may be said that the p_{H} of blood after centrifuging under oil was between 7.7 and 7.8 at 17° , that the haemoglobin appeared to be well oxygenated and that results obtained on normals were consistent and agreed fairly well with those of other observers.

Results.

Normals (Table I, Nos. 1-6). The cases about to be described were either normal or showed a minor degree of anaemia in association with some condition like rheumatoid arthritis. The averages and ranges for the various findings were as follows: haemoglobin 91 % (82-102), erythrocytes 4.95 millions per $\mu\text{l.}$ (4.3-5.4), haematocrit 42.4 % (37-46), mean cell volume $86\mu^3$ (80-91). Haemoglobin per litre of cells 295 g. (272-313). Cell constituents were as follows: K 106 m.eq. (101-110), Na 12 m.eq. (10-15), Cl 51 m.eq. (48-56), water content 71 % (69-72). In the case of the plasma, the figures were: K 4.1 m.eq. (3.8-4.7), Na 139 m.eq. (136-142), HCO_3^- 25.5 m.eq. (23-27), Cl 102 m.eq. (97-106). The ratio of cell to plasma chloride concentrations, R , was 0.65.

Details of individual cases are given in Table I, but it must be noted that cell and plasma constituents are given in terms of their concentrations per litre of water, and if it be desired to convert concentrations into contents per litre of cell or plasma, it will be necessary to multiply the concentrations by the percentage of water present.

The figures for plasma K are a little lower than those of some observers, but agree with those of Laurent and Walther [1935] obtained by a different method. It is possible, that as Walther suggests, the high results of other workers are due to delay in separating cells and plasma.

The average concentrations of cell and plasma base per litre of cell water were respectively 166 and 154 m.eq.—a difference of 12 m.eq. From the latter figures, 3 m.eq. must be subtracted corresponding to an excess of 2 m.eq. cell Mg on the one hand, and an excess of 5 m.eq. plasma Ca on the other. Thus the excess of cell over plasma base is in the present series about 6 %. This excess of cell base is to be expected owing to the presence of the non-permeating Hb⁻ present in the cell. Similarly the sum of plasma $[\text{Cl}^-] + [\text{HCO}_3^-]$ is greater than the sum of cell $[\text{Cl}^-] + [\text{HCO}_3^-]$ being 137 m.eq. compared with 93.

It is generally agreed that the osmotic pressures of erythrocyte and plasma are equal and therefore the sum of cell anion and cation should equal the sum of plasma anion and cation. In the present series the concentration in cell water of $\text{K} + \text{Na} + \text{Cl} + \text{HCO}_3^-$ is 258.5 m.eq. To this figure must be added about 12 m.eq. for cell Hb so that the total osmotic equivalence of the cell contents is about 270 m.eq. The corresponding figure in the case of the plasma is 291. (The osmotic pressure of the plasma proteins which is small has not been considered—for the purposes of the present discussion it may be neglected.) There is thus an apparent excess of at least 21 m.eq. of osmotically active substances in the plasma. Henderson [1928] remarks on this apparent excess of plasma constituents and suggests that it may be due to "differences between the two phases in activity coefficients of water and of dissolved substances" and "to unequal distribution of substances not accounted for in the balance sheet". It is however at least as simple to assume that a proportion of cell water is not free, in the

Table I.

Case No.	Date	Disease	Age	Sex	Haemoglobin %	Erythrocytes millions per µl.	Colour index	Haematocrit	Mean cell vol. µ ³	Haemoglobin g. per litre	Van den Bergh units	Reticulocytes %	Cell concentrations					Plasma concentrations					Excess cell base over plasma m.eq.	Excess plasma osmotic pressure over cell osmotic pressure* m.eq.
													K m.eq.	Na m.eq.	HCO ₂ m.eq.	Cl m.eq.	H ₂ O %	K m.eq.	Na m.eq.	HCO ₂ m.eq.	Cl m.eq.			
1	—	N	24	M.	83	4.32	0.96	39.4	91	291	0.3	0.1	153	14.3	22	67	72	4.3	146	29	104	15	17	
2	—	N	20	F.	97	5.00	0.97	44.1	88	304	0.5	0.2	150	10.6	22	74	71	4.3	152	28	110	10	18	
3	—	N	35	M.	102	5.12	1.00	45.0	88	313	0.5	0.1	147	17.4	20	73	71	5.0	146	26	114	11	22	
4	—	N	16	F.	82	4.50	0.91	37.5	83	301	0.4	0.2	136	15.1	21	71	69	4.0	149	28	113	18	18	
5	—	N	52	M.	91	5.40	0.84	43.0	80	292	0.5	0.1	141	21.5	20	78	71.5	4.5	152	25	113	6	21	
6	—	N	24	M.	90	5.44	0.82	45.7	84	272	0.3	0.2	146	10.6	21	72	71.5	4.6	147	27	108	11	19	
7	—	Mi	60	M.	46	4.03	0.57	29.5	73	215	0.2	1.4	147	17.5	21	71	75	4.5	151	28	109	6	31	
8	—	Mi	42	M.	53	4.12	0.64	29.8	72	245	0.1	0.2	152	20.5	21	74	70	4.0	150	27	110	19	14	
9	26. xi. 35	Mi	63	M.	58	3.3	0.42	18.8	57	205	0.8	2.0	148	18.3	19	70	77	4.9	150	25	111	11	28	
10	14. i. 36	Mi	52	F.	70	5.2	0.67	30.1	75	247	0.3	0.1	152	16.5	20	74	73.5	5.2	152	26	111	11	23	
11	2. xii. 35	Mi	44	F.	43	4.24	0.51	27.0	64	220	0.7	3.0	151	15.2	21	71	73.5	4.0	149	28	112	13	26	
12	30. xii. 35	Mi	44	F.	78	4.9	0.80	38.5	79	279	0.2	0.8	151	10.4	22	71	71.5	4.3	154	28	108	12	20	
13	—	Mi	44	F.	64	4.3	0.51	27.4	64	222	0.5	0	117	17.0	18	63	76.5	4.3	152	26	106	8	35	
14	—	Mi	41	F.	59	5.3	0.62	30.6	69	249	0.3	3.5	150	17.2	21	74	74	4.2	154	26	107	9	20	
15	—	Mi	54	M.	44	5.00	0.44	31.7	63	192	1.5	4.4	156	—	—	—	78	—	—	—	—	—	—	
16	15. xi. 35	Ma	73	M.	44	1.41	1.56	21.0	149	290	1.25	1.9	151	16.0	21	72	79	4.4	150	28	115	13	29	
17	24. xii. 35	Ma	69	F.	72	3.00	1.20	35.0	117	283	0.2	0.5	157	21	20	67	71.5	4.3	158	30	115	16	30	
18	4. xii. 35	Ma	44	F.	43	1.74	1.24	21.2	122	280	2.0	12.5	142	20	18	67	70	4.5	154	28	114	12	24	
19	27. xii. 35	Ma, S	36	F.	51	1.84	1.38	25.7	140	275	3.0	7.0	152	19	16	67	69	4.1	151	26	113	7	35	
20	—	Ma, S	64	F.	84	4.20	1.00	40.3	96	280	0.25	0.6	147	18	22	73	71	4.8	148	29	113	12	23	
21	—	Ma, S	62	M.	42	1.71	1.23	22.9	134	254	0.5	13.0	145	—	—	—	—	—	—	—	—	—	—	
22	—	My	64	F.	40	1.40	1.43	19.7	111	280	—	0	162	—	—	—	—	—	—	—	—	—	—	
23	—	My	56	F.	60	2.80	1.07	29.1	104	285	3.5	11.0	149	17	20	71	69	5.7	153	27	110	7	27	
24	—	AJ	42	M.	94	3.13	0.93	28.5	91	281	2.7	4.8	153	16	19	68	72	4.3	148	26	111	17	21	
25	9. i. 34	AJ	42	M.	94	4.80	0.98	37.3	78	346	—	—	106	—	—	—	—	—	—	—	—	—	—	
26	16. i. 35	AJ	42	M.	90	4.30	1.05	39.0	84	345	9.0	0.0	131	—	—	—	—	—	—	—	—	—	—	
27	11. xi. 35	AJ	42	M.	96	4.05	1.03	37.8	81	351	6.0	5.0	118	20	23	71	65	4.5	154	28	101	—	38	
28	25. iii. 36	AJ	42	M.	89	3.92	1.04	35.2	90	320	6.0	5.0	112	16	17	63	65	4.1	143	24	106	—	53	
29	16. x. 34	AJ	20	F.	78	3.96	0.98	30.2	77	351	4.0	12.0	130	—	—	—	—	—	—	—	—	—	—	
30	2. ii. 35	AJ	20	F.	78	3.75	1.04	32.5	86	331	3.0	10.0	110	—	—	—	—	—	—	—	—	—	—	
31	11. iv. 35	AJ	20	F.	87	4.10	1.06	34.0	83	353	2.5	0.3	126	—	—	—	—	—	—	—	—	—	—	
32	26. ii. 36	AJ	11	M.	86	4.40	0.98	33.0	57	330	3.5	4.0	132	19	20	67	67	4.7	154	28	110	—	40	
33	16. v. 35	AJ	35	F.	55	3.42	0.80	26.4	77	388	3.5	12.0	130	—	—	—	—	—	—	—	—	—	—	
34	26. i. 36†	AJ	60	F.	82	3.97	1.03	34.0	82	326	4.0	12.0	131	22	21	—	—	—	—	—	—	—	—	
35	26. v. 35	AJ	21	M.	105	5.40	1.12	26	108	287	0.4	0.1	136	—	—	—	—	—	—	—	—	—	—	
36	26. i. 36†	AJ	21	M.	87	4.40	0.99	34.5	78	347	5.0	18.0	119	—	—	—	—	—	—	—	—	—	—	
37	26. i. 36†	AJ	21	M.	87	4.40	0.99	34.5	78	347	5.0	18.0	119	—	—	—	—	—	—	—	—	—	—	
38	27. v. 35	AJ	20	F.	106	5.32	1.00	43.7	84	329	0.25	1.4	125	13	21	70	68.5	4.2	145	28	108	—	45	
39	27. v. 35	AJ	20	F.	76	3.62	1.00	31.1	81	339	12.0	18.0	124	18	20	65	68	4.0	149	28	106	—	47	
40	30. i. 36†	AJ	20	F.	110	5.42	1.01	49.6	92	306	1.5	0.4	126	17	22	74	70	4.6	148	27	106	—	34	

N = normal.

Mi = microcytic anaemia.

My = myelodysplasia.

Cell O.P. includes Hb.

Ma = macrocytic anaemia.

S = Sprue.

† Post-operative recrud.

AJ = acholuric jaundice.

N = normal.

Mi = microcytic anaemia.

My = myeloid leucosis.

* Cell O.P. includes Hb.

Ma = macrocytic anaemia.

† Post-operative record.

S = Sprue.

AJ = acholuric jaundice.

sense that it is not available for the solution of substances present in the cell. Thus if 8% of cell water were bound then the discrepancy in the sums of osmotically active substances in cell and plasma would disappear. On general grounds it is to be expected that bound water is present in erythrocytes since as Adair and Adair [1934] have shown 1 g. Hb binds 0.2 g. water and hence the 30 g. Hb present in 100 ml. cells should bind about 8% of the total water present. And whilst on the one hand there is direct experimental evidence that between 5 and 10% of cell water is bound [Maizels, 1935], there is on the other hand no evidence at all that ionic activity is increased in the presence of haemoglobin [Adair and Adair, 1934].

The microcytic anaemias (Table I, Nos. 7-14). The averages and the ranges of the findings in this group are as follows: colour index 0.52 (0.42-0.64), mean cell volume $66 \mu^3$ (57-73), Hb per litre cells 216 g. (180-250), cell K 112 m.eq. (106-122), cell Na 13 m.eq. (11-14), cell Cl 53 m.eq. (48-57), cell water 75% (70-79). Plasma K averaged 4.0 m.eq. (3.7-4.5), Na 141 m.eq. (139-144), HCO_3 25 m.eq. (23-26), Cl 102 m.eq. (99-107), and the ratio of cell to plasma chloride concentrations was 0.64.

Thus the mean cell volume and amount of Hb per unit volume of cells are decreased. In the plasma, K, Na, Cl and HCO_3 are about normal while the contents of cell anion and base are greater than normal. As cell H_2O is also increased however the concentrations of cell acid and base are only slightly raised.

In three of the cases of microcytic anaemia the reticulocyte count was in the neighbourhood of 3%, but this appeared to make little difference to the concentration of cell base. Indeed the latter remained fairly constant in spite of great variations in cell water and Hb in all anaemias with the single exception of acholuric jaundice. It seems as if the concentration of cell base were of the first importance and that the other cell constituents were modified so as to maintain this constancy. Thus in case 9 although, as a result of treatment, Hb rose from 28 to 70% and Hb per unit volume of cells by 20%, the concentration of cell base remained practically unaltered. The same is true of case 10.

The macrocytic anaemias (Table I, Nos. 16-21). One of these cases contained less Hb per unit volume of cells than normal (No. 18), a case of spruce; the others contained about the normal amount.

Colour index averaged 1.36 with a range from 1.24 to 1.56. Mean cell volume was 137 (122-149). The concentration of cell base was about normal, whilst that of anion was slightly decreased. The ratio of cell and plasma chloride concentrations was decreased, being 0.6 against the normal 0.65. Since this ratio varies inversely with cell $[\text{Hb}^-]$ and since the latter is not increased, the decrease in R is difficult to explain, unless some other non-permeating anion be present in the cell besides Hb.

As a result of treatment cell base decreased and water increased, as observed by Henderson in a case described by him. The increase in plasma base noted by Henderson, however, is not observed in this series.

The chloride ratio rises as a result of treatment, but since the concentration of cell Hb does not decrease, the increase in this ratio may perhaps be attributed to a decrease in some other non-permeating anion.

In three cases of the present series the apparent excess of osmotically active substances in the plasma over those in the cell was greater than normal, being 29 m.eq. compared with 21. Henderson [1928] had previously noted this increased discrepancy between plasma and cell osmotic pressures in pernicious anaemia and suggested that it might be due to the presence of reticulated cells

or cells which have recently passed through this stage. In such cells it is possible that "water and electrolytes are associated with nuclear material in proportions quite different from those existing in the cell solution". Equally, it is possible that the reticulocyte has a greater proportion of bound water than has the mature erythrocyte.

But whatever deviation the immature erythrocyte may present, it may be said that cell K remains fairly constant and that cell destruction as shown by increased bilirubin in the blood or cell regeneration as shown by reticulocytosis has very little influence on the potassium level.

Myelosclerosis (Table I, Nos. 21 and 22). The two cases of this rare disease are of interest because of certain findings which sometimes compare and at others contrast with those present in acholuric family jaundice. Features in common are: an anaemia with colour index in the neighbourhood of unity; a marked excess of haemobilirubin in the blood and the presence of nucleated and reticulated erythrocytes.

Myelosclerosis differs from acholuric jaundice in that the amount of haemoglobin per unit volume of cells is normal and not increased and that cell fragility is not increased. In both conditions the spleen is enlarged, but in myelosclerosis splenectomy is not followed by improvement in the anaemia, reticulocytosis or jaundice.

The various chemical findings, including cell potassium are not abnormal in myelosclerosis.

Acholuric family jaundice (Table I, Nos. 23-29). The erythrocyte of acholuric family jaundice (congenital haemolytic icterus) differs from the normal in several respects; it tends to be less biconcave and even biconvex; it contains more haemoglobin per unit volume of cells (345 g. compared with the normal 290). In agreement with this observation cell water is decreased, being 65-69% compared with the normal 69-72%. Mean cell volume is normal or slightly decreased; the decrease in cell diameter is more marked and hence it follows that the cell tends to be more convex than normal.

Cell K is decreased by 20-30%, cell Na is normal; the other constituents of the cell show no abnormality. The decrease of cell K is not related to the intensity of the Van den Bergh reaction or to the reticulocytosis. It would not indeed have been expected to be so related in view of the findings in the anaemias and in myelosclerosis where marked jaundice and noteworthy reticulocytosis are unaccompanied by any significant change in cell K. Nor can cell K be correlated with the cell fragility in saline solutions for in case 25, where haemolysis was seen in 0.7% NaCl, cell K was 87 m.eq., whilst in case 23 with slight haemolysis only in 0.6% NaCl cell K was 72 m.eq. Further the same case may show considerable variations in cell K without corresponding changes in fragility (cases 23 and 24). The findings are summarised in Table II.

The more complete data of cases 23, 24 and 29 show certain other abnormalities. The concentration of cell base (K + Na) is less than the concentration of plasma base, averaging 144 m.eq. against 157, whilst the apparent excess of plasma osmotic pressure is much greater than normal, being over 40 osmolar millimols in each case, compared with the normal 21. Now unless the p_H of the acholuric cell is less than 6.6, the isoelectric point of haemoglobin, cell base concentration must be greater than plasma base. On general grounds the p_H of the acholuric erythrocyte is not likely to be less than p_H 7, in fact although it is more acid than normal its p_H is definitely greater than 7 [Hampson and Maizels, 1927-28]. Here again it may be that, as Henderson suggests, the distribution of electrolytes in the immature erythrocyte is different from that in

Table II.

Case No.	Date	Cell K content m.eq.	Van den Bergh units	Reticulocytosis %	Haemolysis commencing NaCl, %
23	9. x. 34	72	—	—	0.6
	17. xi. 34	72	—	—	—
	16. i. 35	85	9	6	0.6
	30. i. 35	86	—	—	—
	11. xi. 35	77	6	5	0.6
24	16. x. 34	84	4	12	0.57
	22. iii. 35	73	3	10	0.57
	11. iv. 35	82	2.5	6	0.57
	26. ii. 36	87	3.5	4	0.57
25	—	87	3.5	12	0.70
26	16. v. 34	88	4	12	0.51
	3. xii. 35*	92	0.4	0.1	0.48
27	—	82	5	18	0.6
28	26. v. 35	86	9	13	0.57
	26. i. 36*	87	0.25	1.4	0.57
29	27. v. 35	84	12	18	0.6
	20. i. 36*	88	1.5	0.4	0.54

* Post-operative record.

the mature cell. It is however more probable that the increased proportion of bound water in the acholuric cell explains the apparent anomalies. It has been remarked previously that in normal blood, plasma osmotic pressure is apparently greater than cell osmotic pressure, and in order to explain this it was suggested that about 8 % of cell water (6 ml. per 100 ml. cells) was bound. If the same amount of bound water were present in the acholuric cell, cell base concentration would be 150 m.eq. against 157 in the plasma. It is further possible that the amount of bound water in the acholuric cell is greater than that assumed to be present in the normal. This is in fact quite likely, since the absolute amount of haemoglobin in the acholuric cell is about 20 % greater than that of the normal cell, corresponding to the binding of about 10 % of total water. Further the concentration of Hb is also greater and under these circumstances the quantity of water bound is relatively increased. Thus Kunitz *et al.* [1933-34] have shown that 1 g. Hb in 6.3 % solution binds 0.14 g. water, whilst in 10.45 % solution it binds 0.22 g.

It is of course possible that this deficiency in cell K + Na might be compensated by the presence of Ca or Mg, but estimation of total base in one case showed that this was not so.

After removal of the spleen the condition of the patient shows a notable improvement: reticulocytosis and excess of bilirubin disappear from the blood, cell volume increases slightly. The haemoglobin content of the cells falls by 10 %, the water content increases slightly and fragility becomes slightly less marked. [Cell K shows a very slight increase.

DISCUSSION.

There remain for discussion two aspects of the preceding observations: firstly, the relation of cell anion to cell cation, and secondly, the nature of the acholuric erythrocyte.

Relation of cell cation to cell anion. The concentration of cell cation $[K^+] + [Na^+]$ should equal that of $[Cl^-] + [HCO_3^-] + [Hb^-]$. Any excess of cation over the

latter value must presumably be due to the combination of base with some undetermined acid. Such an excess has been demonstrated by Henderson [1928] who refers to the acid as HX. $[X^-]$ equals $[K^+] + [Na^+] - [Cl^-] - [HCO_3^-] - [Hb^-]$. In the present investigations, K, Na, Cl and HCO_3 are known. To determine $[Hb^-]$ it is necessary to know the concentration of Hb and also the cell p_H . The latter value, unfortunately, is not known. It is however possible to assign certain probable limiting values to cell p_H , and hence to $[Hb^-]$ and so to $[X^-]$.

Thus in normals cell p_H will certainly be between 0.05 and 0.2 less than that of the plasma [Hampson and Maizels, 1927-28]. In the present experiments, plasma p_H was between 7.7 and 7.8 and it is very probable that cell p_H was between 7.5 and 7.7. The average concentration of cell Hb was 25.5 m.eq. corresponding to between 52 and 65 m.eq. base bound [Adair, 1925]. The excess of cell base concentration over $[Cl^-] + [HCO_3^-]$ was 72 m.eq. so that the concentration of base uncombined with $[Cl^-] + [HCO_3^-] + [Hb^-]$ was between 7 and 20 m.eq. The latter figures are obviously approximate and it must be interpreted to mean that a small excess of base is present in the normal erythrocyte over and above that combined with $[Cl^-]$, $[HCO_3^-]$ and $[Hb^-]$; it is presumably combined with some other anion of simple or complex nature.

In the microcytic anaemias the value of $[Hb]$ averaged 18 m.eq. The excess of cell base over $[Cl^-] + [HCO_3^-]$ was 75 m.eq. and in the absence of X^- it follows that 4.2 m.eq. base are bound per m.eq. Hb corresponding to a cell p_H of 8.8. Such a low hydrogen ion concentration is quite impossible and it is certain that the anion X^- must be present in the anaemic cell and in greater amounts than in normal cells. It is in fact very probable that p_H 7.5-7.75 will cover the cell p_H range in anaemia and this corresponds to between 38 and 48 m.eq. bound: under these circumstances the concentration of X^- must be between 27 and 37 m.eq. It is clear therefore that when all the various approximations are taken into account, the value of $[X^-]$ in anaemic cells is much higher than in normals. It varied directly with the degree of anaemia and decreased when Hb rose as a result of treatment.

In the macrocytic anaemias, the concentration of base not bound by $[Cl^-]$ and $[HCO_3^-]$ averaged 85 m.eq. and that of $[Hb]$ was 24.6. As the p_H of the macrocytic erythrocyte is less than normal [Hampson and Maizels, 1927-28; Henderson, 1928] it is clear that the base bound by Hb^- will also be less than normal and that X^- will be greater than normal. Thus the p_H difference in pernicious anaemia ranged between 0.2 and 0.5. Assuming then that under the experimental conditions cell p_H lay between 7.25 and 7.55, the concentration of base bound by Hb would lie between 38 and 55 m.eq. and the concentration of X^- between 30 and 47 m.eq.

As a result of treatment the concentration of cell base combined with $[Hb^-] + [X^-]$ fell to 73 m.eq. while the concentration of Hb remained unchanged. Even if one assumes that cell p_H were unaltered (although it had probably in fact actually increased) then it is certain that the amount of base combined with $[X^-]$ had decreased. Such a decrease in the value of X^- in treated cases had been previously noted by Henderson [1928].

The chloride ratio rose in treated macrocytic anaemia. This ratio varies inversely with the concentration of ionised haemoglobin. But as $[Hb^-]$ had not decreased but rather increased, one must ascribe the increase in the chloride ratio to a decrease in some other non-permeating anion, possibly X^- itself.

In acholuric jaundice $[Hb]$ averaged 31.5 m.eq. From the data of Hampson and Maizels, cell p_H may be taken to be between 0.3 and 0.5 less than that of the plasma, namely between 7.25 and 7.45. The concentration of base bound by

$[\text{Hb}^-]$ under these conditions lies between 49 and 63 m.eq. Base combined with $[\text{Hb}^-] + [\text{X}^-]$ equals 55 m.eq. and therefore the concentration of cell X^- lies between +6 and -8 m.eq. From which it follows that the concentration of X^- in this malady is very low.

Regarding the nature of the erythrocyte in acholuric jaundice, it will have been realised that this cell is very distinctive. It tends to be more globular than normal, contains more haemoglobin and less water than the normal cell and is more prone to haemolysis. Its Na content is normal, but K and therefore total base are low. It might be thought that the globular shape of the acholuric cell and its fragility depended on its distension by the increased quantity of the contained Hb but this is probably not the case; thus No. 25 which has the lowest Hb per litre of cells has the highest fragility.

It is probable that the low level of the cell K in acholuric jaundice is significant, although it does not parallel cell destruction, regeneration or fragility and is therefore not dependent on these: like them, however, it may arise from some primary defect in the cell which affects these manifestations in different degrees.

Considerations of such a defect must necessarily be speculative; it might lie in the structure or composition of the cell membrane and might act in one of two ways: (1) accelerated loss of K from the mature cell; (2) delayed permeation of K into the immature cell.

(1) Cell base is chiefly K; plasma base Na. It is possible that the originally impervious cell suspended in plasma, gradually becomes effete and sheds a proportion of its K. Indeed it is possible that haemolysis may occur when cell K reaches a certain low level. There are, however, certain objections to this theory. If K diffuses out of the cell into the plasma, Na should pass from plasma to cell; there is no evidence that this occurs. Further, there is no evidence that the acholuric cell suspended even in simple salt solutions is more pervious than normal. Thus the gain of K in KCl or loss in NaCl solutions (after suspension for 1 hour at 37°) is not greater than normal, although acholuric cells are specially liable to damage by foreign solutions. Finally, it has already been noted that the excessive haemolysis of pernicious anaemia and myelosclerosis is not accompanied by a decrease in cell K. It seems improbable therefore that the low level of cell K in acholuric jaundice depends on accelerated loss.

(2) The level of K in the normal erythrocyte is higher than that of most other cells. It seems probable that the primitive erythrocyte contains no more K than other cells, but that at an early stage an inflow of K occurs, determined by unknown factors. Later, the erythrocyte becomes impervious to all cations, and the entry or exit of K and Na ceases.

This may be part of the process of maturation and if the process occurs too early the content of K achieved will be less than normal so that a number of cells will be left in a more or less immature condition with deficient K and a persistent reticulum in relation to which there exists an excessive proportion of bound water.

SUMMARY.

1. The concentration of anions and cations in erythrocytes is less than the concentration of anions and cations in plasma. An apparent deficiency in cell osmotic pressure thus exists, which makes it necessary to assume that about 8% of cell water is bound.

2. An amount of base is present in the erythrocyte which is greater than that required to combine with cell $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$. It is suggested that this excess of base is combined with some other anion present in the cell. This unknown anion is increased in most anaemias, but not in acholuric family jaundice.

3. Anion, cation and water are about normal in the macrocytic anaemias and in myelosclerosis. In microcytic anaemias, the cation content of the cell and also its water content are increased, but the cation concentration is only slightly raised. The concentration of cell cation is thus relatively constant in spite of great variations in the concentration of cell Hb and notwithstanding wide variations in blood regeneration and destruction.

4. In acholuric family jaundice, cell K and water are low; cell Na is normal and Hb per unit volume of cells is increased. Cell base concentration is less than plasma base concentration in spite of the p_H of the cell being greater than the p_H at which oxyhaemoglobin is isoelectric. This can only be explained on the assumption that more bound water is present in the acholuric cell than in the normal.

5. The level of cell K in acholuric jaundice is not related to cell destruction, regeneration or fragility. It is suggested that it may be due to failure of K to penetrate during an early stage of the cell's development.

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CXXI. THE COMPOSITION OF SOME VEGETABLE FIBRES WITH PARTICULAR REFERENCE TO JUTE.

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THE vegetable fibres commonly met with find very diverse uses according to their physical characteristics and chemical composition. Very few comparable analyses are, however, to be found in the literature, and the wide differences which exist in composition are not generally known. Cross and Bevan [1895] originally devised methods for the study of many fibres and carried out much experimental work on jute. Jute they considered to be a typical example of the "lignocellulose" group of "compound celluloses" and a very convenient material for the investigation of the cellulose-lignin relationship in the cell wall and the chemistry of lignin itself. In some respects their views are not acceptable today, and particularly is this the case with the concept of "compound celluloses". Analytical methods too, have been improved or their shortcomings recognised. Methods which have recently been devised for whole plant materials are applicable directly to a study of isolated fibres.

Through the courtesy of Dr S. G. Barker, and members of the Indian Jute Mills Association, samples of jute of known history and quality, covering the entire commercial range, were available (Jute 6-22). For other samples, I am indebted to Messrs Darragh Smail and Co. Ltd. (coir), the Linen Industry Research Association (flax), Messrs F. Ashton (hemp, manilla hemp, sisal), the Imperial Institute (sisal, jute), Messrs Landauer and Co. (ramie), Messrs Myles and Stewart (jute), Messrs Wigglesworth and Co. Ltd. (hemp), the New Zealand Government (*Phormium tenax*), and Dr S. G. Barker (Pita grass, Chinese hemp, coir and *Calatropis gigantea*).

METHODS.

The following determinations were made on samples previously ground, wherever possible, in a high speed mill. (The coir samples were very resistant and in this case the determinations had to be carried out on material of very variable particle size.) The method of sampling adopted was to select at random a considerable number of small groups of fibres, which, collected together, formed the composite sample for analysis.

I. *Cellulose* by the Norman and Jenkins [1933] modification of the Cross and Bevan [1918] procedure. Chlorination is carried out in solution by acidified hypochlorite. No pretreatment is given, owing to serious effects on one component of the cellulose aggregate.

II. *Lignin* by the Norman and Jenkins [1934] modification of the Ost and Wilkening [1910] process employing 72% H_2SO_4 . The modification involves the pretreatment of the material with 5% H_2SO_4 for 1 hour to remove pentose-containing polysaccharides, it being found that pentose groups in the presence of strong acid slowly give rise to furfuraldehyde, which condenses with the lignin.

III. *Furfuraldehyde yield* by distillation with 12 % HCl and precipitation as the phloroglucide. The yield of furfuraldehyde from the isolated cellulose is also determined.

IV. *Uronic anhydride content* by the yield of CO₂ on boiling with 12 % HCl. This determination was made on only a few samples.

The analytical figures expressed on the oven-dried material are given in Table I.

Table I. *Analyses of some vegetable fibres.*

All results expressed on 100 g. oven-dried material.

No.	Fibre and description	Cellu- lose	Lignin	Total furfur- ald. yield	Cellu- lose furfur- ald. yield	Xylan in cellu- lose	Furfur- ald. from hemi- cell.	Uronic acid anhy- dride
F. 1	Flax (Sandringham)	92.26	6.04	1.86	1.37	2.37	0.5	—
F. 3	Flax (Irish)	91.20	3.27	2.32	1.93	3.01	0.4	—
Cg. 1	<i>Calotropis gigantea</i>	91.10	0.67	1.99	1.19	1.85	0.8	—
R. 1	Ramie 1	85.97	1.01	2.39	0.84	1.30	1.6	—
R. 2	Ramie 2	84.12	1.26	2.39	0.78	1.21	1.6	—
H. 1	Hemp (Italian)	89.23	5.32	1.11	1.12	1.73	0.0	—
H. 6	Hemp (Czechoslovakian)	81.63	5.51	3.13	0.99	1.54	2.1	—
H. 5	Hemp (Chinese)	75.86	4.12	3.87	2.47	3.84	1.4	—
H. 2	Hemp (Indian)	77.27	7.28	2.83	1.15	1.79	1.7	—
Pg. 1	Pita grass 1	90.26	3.13	9.73	7.78	12.07	2.0	—
Pg. 2	Pita grass 2	90.83	2.40	9.75	7.61	11.80	2.1	—
J. 1	Jute (lightening)	74.95	11.70	10.60	7.34	11.39	3.3	—
J. 2	Jute (Dacca Tossa)	78.02	11.22	10.96	7.67	11.00	3.3	—
J. 3	Jute (ordinary Tossa)	75.84	11.29	9.76	7.64	11.85	2.1	—
J. 4	Jute (Daisce)	74.56	11.38	9.75	7.43	11.53	2.3	—
J. 15	Jute (best Jat Suti)	74.20	11.01	9.72	7.16	11.12	2.6	4.00
J. 16	Jute (good Jat Suti)	70.87	12.08	10.00	7.39	11.47	2.6	5.12
J. 18	Jute (average Jat Suti)	73.59	11.26	10.19	7.49	11.62	2.7	5.04
J. 17	Jute (good Suti-Eastern)	74.05	12.02	9.64	7.26	11.26	2.4	4.80
J. 19	Jute (average Suti-Eastern)	69.81	12.64	9.93	7.68	11.93	2.3	6.32
J. 22	Jute (discoloured Suti)	72.55	12.35	10.04	7.05	10.94	3.0	5.04
J. 21	Jute (average rejection Suti)	67.61	14.05	10.30	7.85	12.17	2.5	6.68
J. 20	Jute (stick rejection Suti)	64.78	13.79	10.00	6.90	10.72	4.1	7.20
J. 13	Jute (good soft district Tossa)	74.40	10.37	9.56	7.76	12.04	1.8	4.60
J. 14	Jute (average soft district Tossa)	74.32	11.53	11.07	8.27	12.82	2.8	4.72
J. 6	Jute (good Jat Bogi)	74.54	10.57	10.62	7.48	11.61	3.1	4.36
J. 7	Jute (medium Jat Bogi)	71.91	12.12	10.27	7.99	12.40	2.3	5.50
J. 10	Jute (low Bogi)	73.96	11.79	10.01	8.01	12.42	2.0	4.80
J. 8	Jute (low soft district Bogi)	76.00	11.22	11.17	8.25	12.80	2.9	4.84
J. 9	Jute (very low soft district Bogi)	71.80	13.91	10.00	7.70	11.94	2.3	5.68
J. 12	Jute (average Bogi rejection)	71.87	13.10	10.69	7.51	11.65	3.2	5.28
J. 11	Jute (Bogi stick rejection)	68.74	12.47	10.47	7.32	11.36	3.2	6.36
MH. 1	Manilla hemp	74.14	8.51	9.07	9.04	14.01	0.0	—
Pt. 1	<i>Phormium tenax</i>	72.04	11.13	13.33	9.74	15.09	3.6	—
S. 1	Sisal 1 (African)	74.95	6.04	13.63	11.97	18.56	1.7	—
S. 2	Sisal 2	79.43	6.57	12.81	10.54	16.35	1.3	—
C. 1	Coir (bristle fibre—good)	55.69	30.59	14.98	8.52	13.21	6.5	—
C. 2	Coir (bristle fibre—bad)	51.28	29.75	13.62	6.65	10.32	7.0	—
C. 3	Coir (mattress fibre—good)	54.48	32.63	13.92	9.08	14.08	4.8	—
C. 4	Coir (mattress fibre—bad)	56.97	31.50	14.18	8.91	13.80	5.3	—
C. 5	Coir (Cochin fibre—good)	54.29	28.56	13.90	8.44	13.07	5.5	—
C. 6	Coir (Cochin fibre—bad)	50.52	29.45	13.85	7.48	11.64	6.4	—

INTERPRETATION OF ANALYSES.

I. *Cellulose*. Cotton cellulose, which is taken as the standard for all work on cellulose fibres, is unique in two respects. In the first place, a pure product may be obtained by very mild treatments, since it is neither encrusted nor infiltrated with other constituents, and in the second place, the cellulose itself corresponds

more or less to chemically "ideal" cellulose, being composed solely of polyglucose anhydride molecules. The ordinary cellulosic framework of plant materials, woods and fibres is encrusted with other cell wall constituents, and while consisting largely of "true" cellulose, as typified by that of cotton, is not exclusively so and may contain polysaccharides of other sugars. Such associated polysaccharides or celluloses are retained tenaciously and must be regarded as an integral part of the cellulosic aggregate.

The cellulose associated with most celluloses is xylan, though in Gymnosperms mannan also is found. The nature of the association between cellulose and celluloses is under investigation and will be reported elsewhere. The molecule of cellulose polysaccharides is undoubtedly very much shorter than that of cellulose, and their presence has a considerable influence on the properties of the cellulose aggregate as a whole. The xylan content is obtained from the furfuraldehyde yield of the isolated cellulose by reference to Kröber's tables.

II. *Lignin*. The determination of lignin is deceptively difficult, and a method satisfactory for all types of material has yet to be elaborated. The principle of all procedures is the same—to dissolve cellulose and other polysaccharides by strong acid and to weigh the residue after hydrolysis as lignin. Two serious sources of error exist in the determination, due to the presence of pentose groups in the polysaccharides and to the presence of proteins. The disturbance caused by pentose is minimised by the hydrolytic pretreatment with 5% H_2SO_4 which removes the disturbing polysaccharides, and that caused by proteins did not arise in these fibre samples. The results given may therefore be regarded as approximately accurate and comparable.

III. *Furfuraldehyde yield*. The furfuraldehyde obtained on distillation with 12% HCl may come from two groupings, pentoses and uronic acids. In such materials as were used in this work, the uronic acid content (in encrusting hemicelluloses and pectin) is relatively low, so that the major part of the furfuraldehyde arises from the xylan in the cellulose and the pentose units in the encrusting hemicelluloses or polyuronides. The difference between the total yield and that from the cellulose therefore represents that from the pentose and uronic groups in the encrusting hemicelluloses and pectin, if present. No satisfactory method of direct determination of the encrusting hemicelluloses is known. All methods so far suggested, whether dependent on hydrolysis or extraction, include to a greater or lesser extent the cellulose fraction of the cellulose and are therefore objectionable. Until a procedure which distinguishes between these two important polysaccharide groups is elaborated, the best that can be done for comparative purposes is to take the furfuraldehyde yield of the encrusting hemicelluloses as a measure of the total amount present. No extra precision is gained by calculating separately the furfuraldehyde from uronic acid groups, unless the material happens to have a high content of pectin or polyuronides, a circumstance that was not met with in the fibre samples described here.

IV. *Uronic acids*. Uronic acid anhydride groups are found in pectin and the encrusting hemicelluloses as mentioned above. In retted fibres the pectin content is quite small and the CO_2 yield is therefore almost exclusively due to hemicelluloses.

DISCUSSION OF RESULTS.

Flax, ramie, *Calatropis gigantea* and Italian hemp are fibres with very high cellulose contents and contain only small amounts of lignin. The yields of furfuraldehyde which they give are also low, being mainly derived in flax and hemp from xylan in the cellulose with the implication that in these fibres there

can be little encrusting hemicellulose. Ramie, with a lignin content of about 1 %, contains considerably more hemicellulose, as the major part of the furfuraldehyde is not associated with the cellulose. Chinese hemp and Indian hemp have lower cellulose and higher hemicellulose contents than Italian hemp, which represents the best quality fibre of this group. The remaining fibres may be clearly distinguished from those already mentioned by the relatively high content of xylan which is to be found in the cellulose. Roughly then, isolated vegetable fibres seem to fall into two groups, those which have a high content of cellulose, free or almost free of xylan, and those which contain appreciable amounts of other constituents such as lignin and hemicelluloses, and the celluloses of which contain much xylan. Jute, sisal, manilla hemp and coir are the most important members of the second group. The distinction between these two groups is not sharp in respect to cellulose content, though, with the one notable exception of Pita grass (a Brazilian fibre from the leaf of an agave), the members of the first group tend to be higher in this constituent. With lignin the reverse is the case, Pita grass again being an exception. The lignin of some members of the second group, particularly jute, seems to be intimately concerned with the fibre structure, whilst in the first group, a portion of it at least arises from woody elements not wholly removed in isolation. Flax and ramie, for example, give only weak general colour reactions for lignin.

Whereas in most cases the fibre samples analysed were supplied as typical, in the case of jute and coir, fibres of various qualities and sources were examined. The grading of jute depends almost solely on appearance, which may not be related directly to the amounts of the chief structural constituents present. The grading of coir, on the other hand, is based on the suitability of the fibres for certain rather different uses and is based on stiffness or flexibility. In jute, no clear differences in analysis were found between the two main types, *Corchorus capsularis* ("white jute") and *C. olitorius* ("brown jute"—the Bogi and Tossa samples), but within each group the better samples tended to have the higher cellulose contents. The uronic acid present, being a measure of encrusting hemicelluloses, increased considerably as quality decreased, probably indicating indifferent retting and poor separation from the softer tissues, or over exposure to sunlight with the production of oxycellulose. The xylan in the cellulose of jute, expressed as a percentage of the whole fibre, varied surprisingly little (10.72 to 12.82 % being the limits). Omitting "rejection" and discoloured samples the average analysis of jute is cellulose 73.9 %, lignin 11.6 %, xylan in cellulose 11.9 %. No generalisation is possible from the results obtained on the coir samples, the lignin content of which was higher than that of many woods. The variation in amount of xylan in cellulose was greater than expected in view of the fact that each of the grades of coir fibre comes from the same part of the plant, namely the husk. The figures for furfuraldehyde from hemicelluloses indicate the presence of considerable amounts of this group, approaching the quantity found in mature straws.

In Table II the xylan content of the cellulose of all the samples is calculated as a percentage of the total cellulose containing it. The xylan must not be considered as an impurity or contaminant in the cellulose but as an integral and structural part of the cellulosic fabric forming the fibre bundles. The relationship of the cellulosan fraction to the cellulose containing it is under investigation by Astbury and Norman (unpublished). The view has been put forward that the xylan molecules in the cellulose participate in the micellae, are oriented in the same direction as the cellulose chains and are retained by the same forces as are responsible for the lateral stability of the cellulose chains. The molecules of xylan

Table II. *Xylan content of fibre celluloses.*

Expressed as % on the cellulose.

Fibre	Xylan	Fibre	Xylan
Flax 1	2.6	Jute 21	18.0
Flax 3	3.3	Jute 20	16.5
<i>Calotropis</i>	2.0	Jute 13	16.2
Ramie 1	1.5	Jute 14	17.2
Ramie 2	1.4	Jute 6	15.6
Italian hemp	1.9	Jute 7	17.2
Hemp 6	1.9	Jute 10	16.8
Chinese hemp	5.0	Jute 8	16.9
Indian hemp	2.3	Jute 9	16.6
Pita grass 1	13.4	Jute 12	16.5
Pita grass 2	13.0	Jute 11	16.5
Jute 1	15.2	Manilla hemp	18.9
Jute 2	15.2	<i>Phormium tenax</i>	20.9
Jute 3	15.6	Sisal 1	24.7
Jute 4	15.5	Sisal 2	20.6
Jute 15	15.0	Coir 1	23.8
Jute 16	16.2	Coir 2	20.1
Jute 18	15.8	Coir 3	25.9
Jute 17	15.2	Coir 4	24.2
Jute 19	17.1	Coir 5	24.2
Jute 22	15.1	Coir 6	23.0

are undoubtedly much shorter than the long cellulose chains which may contain 100–300 glucose units. The presence of the xylan in the cellulose must have an important influence on the properties of the aggregate, so that a cellulose high in xylan might be expected to be less perfect in many respects than pure cotton cellulose. It is significant in this connection that the high quality textile fibres are those low in xylan, and that between the two groups there is almost as wide a gap in valuation as in xylan contents. The difference is also reflected in commercial uses and market prices.

The highest contents of xylan in cellulose have been found in cereal straws and hard woods (*vide* Table III), the celluloses of which are notoriously of poor quality. Coir cellulose seems to be similar in composition to the straws. No claim

Table III. *Xylan content of some other celluloses.*

Expressed as % on the cellulose.

Material	Xylan
Oat straw	29.3
Barley straw	23.5
Wheat straw	28.6
Rye straw	29.4
Oak wood	26.5
Beech wood	24.0

is made that there is an absolute relationship between xylan content and quality, but only that the xylan content is an indication of the type of cellulose present. The percentage of xylan in the cellulose of any given material is not constant, but varies, within limits, with age and environmental conditions. Xylan has been found in the cellulose of extremely young cereal seedlings, but is present to a greater extent in cellulose produced later. It is presumably laid down by the same mechanism as synthesises true cellulose, the balance between the two components changing with age.

From the practical aspect, the division of fibres into two broad groups on the basis of xylan content is of some importance since statements have frequently

been made to the effect that if certain coarser fibres could only be delignified and softened, they could replace the more expensive fine fibres. Even if this were achieved the celluloses would be of different types and possess different properties.

The extraction and hydrolysis of celluloses containing a high percentage of xylan has been investigated and will be reported elsewhere. Such celluloses are far more susceptible than cotton. Apart from the loss of xylan on treatment with acid or alkali, a portion of the cellulose, or a hexosan fraction associated with the cellulose, comes into solution concurrently. The existence of a less resistant portion of the cellulose has long been known, and in the arbitrary method of estimation of α -cellulose (cold extraction with 17.5% NaOH) the β - and γ -fractions together represent approximately the amount of xylan *plus* less resistant hexosan. The figure is only approximate since the α -cellulose residue is rarely pentose-free. The nature and amount of the less resistant hexosan fraction in fibre celluloses is a subject calling for investigation. Some observations that have been made on the effect of hot dilute alkalis on fibre celluloses show that generalisation is not possible. The celluloses were prepared and extracted without drying

Table IV. *Extraction of celluloses with hot alkali.*

Results expressed as % oven-dry cellulose.

	Conc. of NaOH %	Residue	Xylan in residue	Xylan loss	Hexosan loss	$\frac{\text{Xylan loss}}{\text{Hexosan loss}} \times 100$
Flax	0.1	94.9	2.5	0.0	5.1	—
	1.0	86.6	1.6	0.9	12.5	7
Ramie	0.1	98.1	1.5	0.0	1.9	—
	1.0	90.7	0.9	0.7	8.6	8
Italian hemp	0.1	96.4	1.3	0.6	3.0	20
	1.0	85.5	1.2	0.7	13.8	5
Jute	0.1	94.2	12.4	2.3	3.5	40
	1.0	88.7	10.8	3.9	7.4	35
Manilla hemp	0.1	95.1	15.8	2.6	2.4	52
	1.0	93.3	14.8	3.5	3.2	53
Sisal	0.1	92.7	16.3	4.0	3.3	55
	1.0	90.7	14.4	5.9	3.3	64
Barley straw	0.1	82.2	12.3	12.3	5.5	69
	1.0	73.4	8.5	16.1	10.5	60

with 0.1 and 1.0% NaOH at the boiling-point for 1 hour (Table IV). The high-xylan fibres are not notably more affected by these extractions than the low-xylan fibres. In the former group, jute cellulose stands out from manilla hemp and sisal in that the xylan removed forms a lower proportion of the total loss, and that 1.0% NaOH removes a considerably greater amount of the less resistant hexosan than does 0.1%, whereas in the other two cases little difference was found. The barley straw cellulose, typical of all cereal celluloses, is far more susceptible to extraction, a high proportion of the loss being xylan. That jute cellulose contains more of a hexosan fraction easily removed by alkali than sisal or manilla hemp celluloses may also be seen from Table V. Samples were treated in the cold with 0.5 to 10% NaOH. From sisal or manilla hemp, concentrations above 4% result in the removal of little additional hexosan material. In jute, the amount increases progressively with concentration.

The experiments described here on the effect of alkali on fibre celluloses show the extreme variability in properties that occurs and also that it is not possible to

Table V. *Extraction of fibre celluloses with cold NaOH.*

2 hours—room temperature.					
Results expressed as % oven-dried original cellulose.					
Alkali conc. %	Total loss	Xylan in residue	Xylan loss	Hexosan loss	Xylan loss Total loss $\times 100$
<i>Jute:</i>					
0.5	3.52	13.50	1.23	2.29	35
1.0	4.62	13.31	1.42	3.20	31
2	5.74	12.29	2.44	3.30	43
3	10.40	9.60	5.13	5.27	49
4	13.48	7.70	7.03	6.45	52
5	15.13	6.01	8.72	6.41	58
6	16.95	4.35	10.38	6.57	61
7	21.00	2.63	12.10	8.90	58
8	21.28	2.71	12.02	9.26	57
9	22.61	2.56	12.17	10.44	54
10	23.03	2.50	12.23	10.80	53
<i>Sisal:</i>					
0.5	2.16	18.93	1.43	0.73	66
1.0	3.26	18.19	2.17	1.09	67
2	7.40	15.79	4.57	2.83	62
3	11.26	12.84	7.52	3.74	67
4	15.65	9.13	11.13	4.52	72
5	17.22	7.92	12.44	4.78	73
6	18.60	6.40	13.96	4.64	75
7	19.70	5.49	14.87	4.83	76
8	19.76	5.12	15.24	4.52	77
9	20.10	4.56	15.80	4.30	79
10	20.32	4.25	16.11	4.21	79
<i>Manilla hemp:</i>					
1.0	3.95	14.39	3.96	—	100
2	7.39	12.49	5.86	1.53	79
3	10.13	9.31	9.04	1.09	89
4	12.81	7.68	10.67	2.14	84
5	14.59	6.68	11.67	2.92	80
6	15.41	6.03	12.32	3.09	80
7	16.40	4.88	13.47	2.93	82
8	17.90	3.69	14.66	3.24	82
9	18.49	2.51	15.84	2.65	85
10	19.42	1.41	16.04	2.48	87

deduce from xylan content the resistance of the cellulosic aggregate. It has sometimes been the practice to correct the gross cellulose figure for pentosan content to obtain "true" cellulose or "pentosan-free cellulose". The validity of such a correction is questionable, not only on structural grounds but because the residues may apparently contain varying amounts of a less resistant hexosan fraction. The molecular size of the true cellulose chains in these fibres may also be more variable or shorter than those of cotton cellulose, though the evidence on this point obtained by viscosity measurements is not conclusive, as the effect of the other components has not always been given adequate consideration. In any examination of the factors affecting the properties of a cellulosic fibre these points of ultimate composition and micellar structure must be taken into account as well as the nature and distribution of encrusting substances.

SUMMARY.

Vegetable fibres of many types fall into two well-defined groups, according as the cellulose of the fibre is low or high in xylan. The first group, low in xylan, includes the high grade fibres such as flax, ramie and Italian hemp. The

second group, high in xylan, consists of fibres of the coarser type, such as jute, manilla hemp and sisal, all of which contain also appreciable amounts of lignin and encrusting hemicelluloses. No direct relationship between quality and xylan content was found in a wide range of jute samples. The resistance or susceptibility of isolated cellulose to such treatments as boiling with dilute alkalis cannot be deduced from the xylan content, owing to the presence of varying amounts of easily extractable hexosan.

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CXXII. THE EFFECT OF VITAMIN A DEFICIENCY ON THE DEVELOPMENT OF THE RETINA AND ON THE FIRST APPEARANCE OF VISUAL PURPLE.

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ONE of the earliest effects of vitamin A deficiency is a failure in the regeneration of visual purple in the retina [Fridericia and Holm, 1925; Tansley, 1931; Yoshiere, 1925]. The deficiency may also cause damage to the outer limbs of the rods which contain the visual purple [Tansley, 1934]. It was thought, therefore, that by depriving very young animals of vitamin A during the period when the retina, and particularly the outer limbs of the rods, are still developing, it might be possible, not only to inhibit the production of visual purple, but also to produce retinæ with abnormal, or perhaps no, outer limbs to their rods.

Rats were chosen for this study partly because much is known about their vitamin requirements and partly because their eyes only become mature at a relatively late date after birth. The retina is not fully developed until the rat is 15 days old and visual purple does not appear until at least 12 days after birth and often later [Tansley, 1934]. Dann [1932] found that the young rats of mothers deprived of vitamin A possessed no reserves of the vitamin in the liver at birth. It was, therefore, thought that it might be possible to obtain animals which were deficient in vitamin A during the crucial first 2 weeks of life when the outer parts of the retina are developing.

Methods.

The mother rats were taken from the Lister Institute breeding stock. This stock has an exceptionally good diet and therefore the mothers probably had adequate vitamin A reserves at the beginning of the experiments. The vitamin A-deficient diet used subsequently was that usually employed at the Lister Institute. It was made up as follows:

Caseinogen (Prideaux's)	100 g.
Wheat starch	250 „
Hardened cottonseed oil (irradiated)	75 „
Salt mixture	25 „
Dried yeast	92 „
Lemon juice	25 ml.
Potassium iodide (6.4 g. per litre)	0.33 „
Copper sulphate (15.25 g. per litre)	1.0 „
Water	350 „

Three series of experiments were done. In the first the mothers on the ordinary stock diet were mated at about 200 g. weight and received the deficient diet in the 2nd week of pregnancy. They were then about 4 months old. No

¹ Working with a part time grant from the Medical Research Council.

vitamin A was given during the latter half of the pregnancy period or during lactation. The rats were allowed to suckle the whole litter. In the second series the mothers were given the deficient diet when very much younger, as soon as they had reached about 60 g. weight. This would be at about 5-6 weeks old. After 4 weeks, or sooner if they began to decline in weight, 1 γ β -carotene in coconut oil was given daily; this dose is just sufficient to maintain non-pregnant rats on the deficient diet described above. These animals were allowed to mate as soon as they would after going on the deficient diet. This occurred 8-34 days later but most often after 15-20 days. In the third series the procedure was the same as in the second, except that 2 γ β -carotene daily were allowed. In both series II and III the carotene was replaced by 2 g. daily of wheat germ as soon as the placental sign appeared (usually about the 10th-15th day of pregnancy). This material contains only small amounts of vitamin A but has been found to assist lactation and in series III it was continued until the young were weaned. In this series only two young rats from each litter were left with the mother.

In series II and III vaginal smears were taken daily from the mother rats both before mating and during pregnancy.

The young were killed at varying ages during lactation and their eyes were sectioned and stained in order to detect the characteristic changes found to occur in the retina in vitamin A deficiency. These changes are shown in a shortening and ragged appearance of the outer limbs of the retinal rods together with a marked thinning at the junction of the outer and inner limbs. There is also a failure to produce visual purple which may be detected by fixing the eyes in platinum chloride. This fixative colours the visual purple in the rods yellow and no yellow reaction is given by the retinae of vitamin A-deficient animals [Tansley, 1934]. In the case of animals killed at 15 days of age or older, one eye was treated with platinum chloride as a test for the presence of visual purple.

Those young rats which were reared to the stage of weaning then received the vitamin A-deficient diet until they showed a serious decline in weight. Some were then killed and their eyes examined, while litter-mates were given cod-liver oil until the weight began to recover, when these were also killed and the eyes investigated.

The eyes of control young rats from the Lister stock were also examined at 12 and 15 days of age in order to confirm the normal course of development of visual purple and of the rods in the retina.

The fixatives used were Zenker, Bouin and Flemming without the acetic acid (F. W. A.), and the stains were Mallory's triple connective tissue stain and Mallory's phosphotungstic acid haematoxylin. Stern's [1905] method was used as the test for visual purple.

Results.

Series I. In three cases the experiment failed. In one case the mating did not result in pregnancy and in two cases the mother rats would not eat enough of the deficient diet. One of the latter cast a litter of 10 of which 8 were dead, and failed to suckle the remaining 2. The other was transferred to the stock diet 5 days before parturition; 6 of the litter of 11 were born dead, but the other 5 were satisfactorily reared and all their retinae developed normally.

Four rats produced and suckled litters of 9-13 young. Although of normal weight at birth, these young were all under weight at weaning, the average weight at 21 days being only 27 g. as compared with about 40 g. for the normal young of this stock. The results of the histological examinations of the retinae are given below with those of series III.

Series II. Fifteen rats became pregnant but none was successful in casting and rearing her litter. All these rats had an abnormally long gestation period and none took less than 6 hours over the actual labour. The normal gestation time in the rat is 21–22 days and labour should take about 2 hours. Of the above 15 rats, 1 cast a litter on the 22nd day of pregnancy, 2 on the 23rd day, 5 on the 24th day, 3 on the 25th day, 3 on the 26th day and 1 on the 28th day. One rat was 6 hours in labour, 8 took 12–24 hours, 3 over 24 hours and 1 took 2 days. Two rats died after 2 days in labour, having produced dead litters of 2 and 3 respectively and having several foetuses still in the uterus. The 15 rats produced at least 99 young, of which 85 were born dead and 14 alive. The figure for the dead young is probably too low since nearly all the bodies were partially eaten when found and it is likely that some were entirely destroyed. The average size of litter was, however, much below normal. Two of the mother rats died 3 and 4 days after the uterus was emptied. None of the young which were born alive survived more than 2 days.

This condition seems to be due, at least in part, to uterine inertia. In several cases the uterine muscle was found *post mortem* to be quite flaccid. The symptoms are probably due to the vitamin A deficiency, since a daily dose of 2γ β -carotene, as given to the mothers in series III, was found to prevent their appearance. Similar results were noticed by Mason [1935] in rats deprived of vitamin A. In some of his rats the deficiency was severe enough to prevent the continuation of pregnancy beyond the 2nd week. Although early death and resorption of all the foetuses was not found in the experiments described above, this probably took place to some extent since at least 9 of the 15 litters were abnormally small.

Series III. Nine rats became pregnant in this series. The resulting litters, which contained an average of 10 young rats, were all cut down to 2. Although, judging by the condition of the retinae, these young were, on balance, more deficient than those of series I, the gain in weight during suckling was better, the weight at weaning being 30–35 g. This indicates that the very poor growth shown by the young of series I was chiefly due to lack of food and not entirely to any transference of vitamin A deficiency from the mother to the young.

In series II and III, where vaginal smears were taken daily until littering, it was found that in no case was the deficiency severe enough to cause a constant appearance of cornified cells. Of the rats in series II, 4 had normal cycles before mating and a constant anoestrous smear after mating. Of the remaining 11 rats, 4 had frequent cornified cells in the vagina both before and after mating though not sufficient to obscure the oestrous cycle. The other 7 rats tended to show a prolonged oestrous smear before mating but were normal afterwards.

In series III all but 2 rats produced normal smears throughout the experiment. Of these 2, one had occasional cornified cells in the smears taken during pregnancy while the other showed a cornified smear lasting 2–3 days instead of the normal 1 day before mating.

A general review of the three series of experiments described above (see Table I) leads one to the following conclusions.

(1) A complete absence of vitamin A from the food of a pregnant rat, which had had a good diet for the first 4 months of life, did not affect its ability to complete a pregnancy successfully. If the deficiency was continued during lactation, however, insufficient milk was secreted for the satisfactory rearing of a large litter. A normal rat of this stock is quite able to rear a litter of 10 to a weight of approximately 40 g. per individual young rat at 21 days. In the cases here studied the milk seemed to be deficient in quality as well as quantity since all the young rats of the first series showed signs of vitamin A deficiency.

Table I. *Summary of history of rats before and after pregnancy.*

Series	No. of mothers	Wt. and age at beginning of exp. g.	Wt. at beginning of pregnancy g.	Diet	No. which reared young	No. of young reared	Av. wt. of young at weaning g.
I	7	ca. 200 (ca. 4 months)	ca. 200	Vitamin A-deficient during second half of pregnancy and lactation	4	41*	27
II	15	ca. 60 (ca. 5-6 weeks)	89-144	Vitamin A-deficient, supplemented after ca. 4 weeks with 1 γ β -carotene daily. Carotene replaced by 2 g. wheat germ after 15th day of pregnancy	0†	0	—
III	9	ca. 60 (ca. 5-6 weeks)	90-143	As in series II but supplement of 2 γ β -carotene instead of 1 γ	9	18‡	30-35

* Litters not reduced.

† All mothers showed prolonged gestation and difficult labour.

‡ Litters reduced to 2.

(2) A small daily ratio of 1 γ of β -carotene, which is sufficient to maintain a non-pregnant rat on a vitamin A-deficient diet was not enough to enable a pregnant animal to cast its litter.

(3) A very small increase in the extra carotene provided, to 2 γ daily, not only allowed normal reproduction to take place but also made it possible for the mother to rear a small litter. The milk, however, did not contain sufficient vitamin A to protect the young from the retinal abnormalities which follow a deficiency in this vitamin.

General histological examination of the young rats. All the young from series I and III, as well as those which were not partially eaten from series II, were well developed and of normal weight (4-5 g.) at birth. The lungs, kidneys and eyes of many of the young from series II and III were examined histologically at birth for signs of keratinisation or abnormal development, and with the exception of one litter from series II, all were found to be normal. The only abnormal litter was one of 11 cast by a rat from series II at intervals in a labour lasting over 6 hours. Of these 7 were born alive and 5 dead, but none survived till the next day. Of the living young 3 were very blue and made violent gasping efforts about once a minute. On examination the lungs were found to be very full of blood and almost solid with a few large cavities. The general appearance suggested that something had prevented the proper expansion of the greater part of the lung with the movements of the chest wall but that the pull had been enough to produce abnormally large cavities where the tissue could expand. The circulation through the lung had obviously been established.

Examination of the retinae of the young rats from series I and III. The eyes of 59 young rats were examined at ages from 3 to 61 days. No changes from the normal development of the retina were detected during the first 12 days of life, that is until the first appearance of the outer limbs of the rods. After this period the development of the outer limbs tended to be retarded but was never prevented altogether. Of 23 rats killed between 12 and 21 days, 12 showed almost normal morphological development of the retina although only 4 had perfectly healthy outer limbs. No visual purple was produced before 21 days of age and even then the amount was far below normal. In normal young rats visual purple can usually be detected between the 12th and 15th days. All the deficient

retinae except those from 2 rats showed the characteristic lack of differential staining of the outer limbs.

Of the 59 young rats suckled by deprived mothers 12 were weaned on to the vitamin A-deficient diet. Of these one developed a small corneal ulcer 36 days after weaning and another severe xerophthalmia of both eyes 35 days after weaning. In all the others the eyes remained healthy externally. Of these 12 rats 5 received cod-liver oil, 2 for 3 days, 1 for 5 days and 2 (of which the young rat with xerophthalmia was one) for 6 days before being killed.

The 7 rats which did not receive cod-liver oil were killed between 44 and 58 days of age. All the retinae showed typical symptoms of vitamin A deficiency with poorly stained, unhealthy outer limbs to the rods and thinning at the junction of outer and inner limbs. Dosing with cod-liver oil for only 3 days was sufficient to restore the staining reactions but even 6 days' treatment was not enough to repair the damage to the structure of the outer limbs. In the rat with xerophthalmia which was killed after receiving cod-liver oil for 6 days the retina had completely disappeared in one eye and was seriously affected in the other. However, in the better eye the rods stained normally where they were still present, indicating that even when the structure of the retina is severely damaged, administration of vitamin A is able to restore the normal staining reactions.

DISCUSSION.

The results of these experiments are rather disappointing since it seems doubtful whether the young rats examined were suffering from a severe deficiency during the actual period of retinal development. The effect of vitamin A deficiency in upsetting the mechanism of labour makes it difficult to obtain young rats seriously deficient at birth while the effect on lactation adds a further complication.

It does appear, however, that partial deprivation of vitamin A will postpone the first appearance of visual purple and, in some cases, prevent its formation in young suckling rats. These experiments also indicate that the first effect of vitamin A deficiency on the developing retina is shown on visual purple formation and that the damage to the rod structure is a later effect. Thus, although visual purple is not found in unhealthy rods [Tansley, 1934], it would seem probable that vitamin A affects the production of visual purple directly rather than by interference with the normal health and structure of the rods. This idea is in harmony with Wald's conception of the visual purple system. He believes that visual purple is formed by a direct combination of vitamin A with protein [1935], and the results described above may certainly be explained on this theory.

SUMMARY.

1. An attempt was made to obtain young rats which were deficient in vitamin A during the first 3 weeks of life.
2. Pregnant rats subjected to too severe vitamin A deficiency were found to show a prolonged gestation period and an abnormal and difficult labour. They also failed to suckle their litters.
3. It was therefore found possible to induce only a moderate, and not a severe, deficiency in young rats during the suckling period.
4. In such young rats the development of visual purple was retarded and in some cases prevented, while the effect on the structure and development of the rods was not so marked.
5. The part played by vitamin A in the visual purple system is discussed.

I have to express my thanks to the Lister Institute for the hospitality shown to me during this investigation; also to Miss E. M. Hume and Miss Henderson Smith for much help and advice in the feeding and care of the rats.

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CXXIII. THE WATER-SOLUBLE B-VITAMINS.

V. NOTE ON THE TWO TYPES OF SKIN LESION OCCURRING IN VITAMIN B₂ DEFICIENCY IN THE RAT IN RELATION TO DEFICIENCY OF FLAVIN AND VITAMIN B₆, RESPECTIVELY.

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IN recent papers György [1934; 1935] has shown the relation of the two constituents of vitamin B₂, flavin and vitamin B₆, to the skin lesions seen in rats receiving diets deficient in vitamin B₂. Chick *et al.* [1935] also described two kinds of skin symptoms:

(a) A symmetrical florid dermatitis, with redness, swelling and oedema affecting first the paws and tips of ears and nose. This condition is similar to the "specific type" of dermatitis described by György and the pellagra-like condition in rats described by Goldberger and Lillie [1926].

(b) An affection of the skin in which the hair is shed without swelling or inflammation, with development of bald patches especially over the head and face: the eyelids are often stuck together with a serous, blood-stained fluid which exudes from the eyes and from the nostrils. This condition corresponds to that described as "non-specific" by György.

György found that vitamin B₆ prevented and cured the "specific", and flavin the "non-specific", skin lesions. Although in this laboratory we found that flavin cured the (b) skin affection, vitamin B₆ did not always cure the florid (a) type of dermatitis and we rather doubted whether the relation of the "pellagrous" rat dermatitis to lack of vitamin B₆ was as simple as György concluded [Chick *et al.*, 1935, pp. 730, 732].

It was, however, realised that the specificity of the two factors for these different skin affections could be better studied by observations of the prophylactic type on young rats maintained from the time of weaning on diets lacking only in flavin or only in vitamin B₆. Subsequently attempts were made to cure the sick animals. These experiments, of which a short summary has already been published [Copping, 1935], form the subject of the present paper.

METHODS.

In previous studies [Chick *et al.*, 1935] on vitamin B₂, a basal diet containing crude maize sugar as a source of carbohydrate produced skin symptoms more regularly than one containing rice starch. The following diet was therefore used: caseinogen 100, maize sugar 300, cottonseed oil 60, lard 15, salt mixture (McCollum's no. 185) 25. The diet was thoroughly mixed by hand and stored dry, but before feeding was made into a stiff paste with distilled water. Vitamins A, D and B₁ were given separately from dropping pipettes, A and D as cod-liver oil, and B₁ as Peters's concentrate from yeast, prepared according to the modified method described by Kinnersley *et al.* [1933]. The rats, which were deprived of

vitamin B₆ only, received 12γ of pure hepaflavin daily, and litter-mates, deprived of flavin only, received doses of vitamin B₆ as 1 ml. (equivalent to 0.5 g. of original dry yeast) of a yeast extract from which vitamin B₁ and flavin had been removed by autoclaving and treating with fuller's earth [see Chick *et al.*, 1935]. In some curative tests, vitamin B₆ was given as a cold alcoholic extract of whole wheat or whole maize, prepared according to the method described by Bourquin and Sherman [1931] for use in a modified Sherman and Spohn diet, in doses equivalent to 4 g. of original cereal [see also Copping, 1936].

Table I. *Type of skin lesions developed on "–B" diet with addition of vitamin B₁ and either flavin or vitamin B₆.*

(a)=florid dermatitis; (b)=non-inflammatory skin affection with loss of hair; (0)=no symptoms.

Group	Component of vit. B ₁ given	No. of rats	Preparatory period				Time in weeks	Av. weekly increase in wt. g.	Curative period				Result
			Type of skin lesions						Curative material added	No. of rats	Time in weeks	Av. weekly increase in wt. g.	
			(0)	(a)	(b)	(a) + (b)							
A	0	3	0	2	1	0	3-5	-0.3	(1) 12γ flavin + purified vit. B ₆ ≡0.5 g. yeast, dry wt. (2) 12γ flavin + alcoholic extract of maize or wheat ≡4 g. cereal	1, (b) 2, (a)	4 1	12 16.5	Cure not quite perfect Perfect cures
B	12γ flavin	10	1	8	0	1	1-14	3.1	(1) Purified vit. B ₆ ≡0.5 g. yeast, dry wt. (2) Unpurified yeast extracts≡0.5 g. yeast, dry wt. (3) Alcoholic extract of maize or wheat≡4 g. cereal	3, (a) 1, (0) 2, (a) 1, (a) + (b)	1-1 3 3-4	-0.7 9.9 11.5	Animals deteriorated Imperfect cures Perfect cures
C	Vit. B ₆ as a purified yeast extract≡0.5 g. yeast, dry wt.	10	3	0	6	1	2-14	2.6	12γ flavin	10	3-4	12.3	5 cures, 3 deteriorated,* 2 died

* These animals were afterwards cured and growth was restored when the purified vitamin B₆ was replaced by an unpurified yeast extract or an alcoholic extract of maize.

In the experiment detailed in Table I, the test animals were three litters of young rats which had been partially deprived of vitamin B₆ from birth. This was done by removing yeast from the diet of the lactating mother and, for the last week of lactation, replacing the stock diet by the usual "–B" diet, supplemented by vitamin B₁ as Jansen and Donath's acid clay adsorbate from rice polishings. At weaning the rats usually weighed 35–40 g. and were immediately placed in separate cages and given the experimental diet and doses of vitamins A, D, B₁ and either vitamin B₆ or flavin. 3 rats received neither component of vitamin B₆ and acted as controls.

Of the 10 rats which received flavin only from the time of weaning, 8 developed the florid (a) type of dermatitis, 1 showed both (a) and (b) symptoms combined, and 1 had no definite skin symptoms at the end of 14 weeks. Six of these animals, derived from two of the litters, were successfully cured by administration of vitamin B₆ given either as an autoclaved yeast extract, or as an alcoholic extract of maize or wheat, although the increase in weight on the combined doses tended to be subnormal, the weekly average gain being 9.9 g. in the former case and 11.5 g. in the latter. The condition of the 4 rats from the third litter was bad after the preparatory period on flavin alone, and only one

cure was successful. The others deteriorated after receiving vitamin B₆ as purified or unpurified yeast extract; 1 died and the other 2 were killed.

Of the 10 rats receiving vitamin B₆, none showed the florid (*a*) type of skin symptoms, 6 developed the (*b*) type, one had a combination of symptoms and 3 showed no definite skin symptoms after 14 weeks on the diet. The condition of the animals receiving vitamin B₆ only was generally worse than that of animals receiving flavin only, even though the skin symptoms were less severe. Two of the rats in this group died shortly after the curative dose of flavin was given, 5 cured very slowly, and in 3 cases definite florid (*a*) symptoms developed within a few days after the flavin dose was given. The reason for this is not clear, but it seems as if flavin, given under these circumstances, may at first cause the pathological process responsible for the florid dermatitis to flare up temporarily, even though vitamin B₆ is present in the diet. The florid symptoms eventually cleared up spontaneously before the end of the experiment, but the cure was slow.

Of the 3 negative control rats which had received no vitamin B₂ component from the beginning of the experiment and were observed for 3 to 5 weeks, 2 showed some type (*a*) symptoms, which were rapidly cured by alcoholic extracts from maize and wheat, respectively, and 1 showed the (*b*) type. All 3 animals finally received flavin and vitamin B₆ and were satisfactorily cured of all symptoms and grew normally.

The results of these experiments show clearly that flavin prevents the (*b*) type and vitamin B₆ the (*a*) type of skin disorder. Further evidence of the effect of flavin in preventing the (*b*) type of skin lesions is recorded in the accompanying paper [Copping, 1936]. In a long series of prophylactic experiments with a modified Bourquin and Sherman diet which contains vitamin B₆ as an alcoholic cereal extract, the type (*a*) dermatitis was observed in only 1 rat, and the type (*b*) was shown by 37 rats. These were rapidly cured and growth was restored by the addition of flavin to the diet. Full confirmation is thus given of the conclusions of György [1934: 1935].

The above experiments also demonstrated the indispensability of both flavin and vitamin B₆ for maintenance as well as for healthy skin condition and suggest that in order to obtain full benefit of the one, the other must be provided in adequate amount. As in our former series [Chick *et al.*, 1935], attempts to cure by flavin or a purified concentrate of vitamin B₆, respectively, were not always successful, except when alcoholic extracts of wheat or maize were used as source of vitamin B₆.

SUMMARY.

1. In a series of prophylactic experiments with young rats maintained on diets lacking only one constituent of vitamin B₂, those deprived of flavin developed the (*b*) type of skin lesions, whilst those deprived of vitamin B₆ developed the florid (*a*) type of dermatitis.

2. In the former case cures were observed and growth restored in 5 rats out of 10 by the administration of pure flavin. In the latter case speedy and complete cures were obtained by administration of vitamin B₆ as an alcoholic extract of whole maize or wheat, but cure was more irregular and imperfect when vitamin B₆ was given in the form of a preparation from yeast.

In conclusion I make grateful acknowledgement of the continued helpful advice of Dr H. Chick and of Sir Charles Martin. I have to thank Dr Sidney Smith for the supply of hepaflavin which enabled this work to be done, and the Medical Research Council for a personal grant.

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CXXIV. THE WATER-SOLUBLE B-VITAMINS.

VI. FLAVIN AND VITAMIN B₆ IN CEREALS.

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IN the course of investigations on the skin symptoms in rats due to lack of vitamin B₂ and of its separate components, it was found advisable to study the effect of the Bourquin and Sherman [1931] type of diet. This diet has been widely used in the study of vitamin B₂, and recently by Kuhn *et al.* [1933, 1]. It was originally devised to be free from vitamin B₂ and to contain vitamin B₁ as an alcoholic extract of whole wheat.

Booher [1933; 1934] demonstrated that when this diet was supplemented with flavin good growth was obtained, but Kuhn *et al.* [1933, 2] and György *et al.* [1934] found that some further factor obtained from yeast, and considered at that time to be vitamin B₄ [Reader, 1929], was required as well as flavin to supplement the diet completely. Nevertheless, flavin was assumed to be identical with vitamin B₂. Later results published by Euler *et al.* [1934] and György [1934; 1935] and also the observations of Chick *et al.* [1935] showed that vitamin B₂ was composite in nature, that flavin was only one component, and that a supplementary substance was also present, which was named vitamin B₆ by György.

In this laboratory preliminary tests with Bourquin and Sherman diets confirmed the observations that the growth of rats could be stimulated when this diet was supplemented with flavin and indicated that while flavin was lacking in the alcoholic cereal extract present in the diet, vitamin B₆ was present in abundance. This was confirmed by Birch and György [1935] and this conclusion is also supported by observations reported in another paper [Copping, 1935; 1936] on the relation between the constituents of vitamin B₂ and the occurrence of different types of skin lesions. Alcoholic extracts of wheat and maize were potent in curing the skin symptoms of the florid (a) type developed in the absence of vitamin B₆ [see Chick *et al.*, 1935].

Considering the dietetic importance of cereals, the connection of endemic pellagra with the consumption of maize and the theories connecting the etiology of this disease with deficiency of vitamin B₂, it seemed of interest to investigate more fully the dietary factors present in an 80 % alcoholic extract of maize and other cereals, and the relative amounts of the two components of vitamin B₂ present in whole maize and wheat and their milled products.

METHODS.

Alcoholic extracts of whole wheat, white patent flour, whole maize, maize endosperm (polenta) and ground polished rice were prepared according to the method of Bourquin and Sherman.

800 g. of the cereal were shaken at room temperature (*ca.* 18°) for 1·5 hours with 1·5 litres of 80 % (by weight) alcohol, and the extract filtered through a Büchner filter with suction. The residue was then shaken for 1 hour with a fresh litre of 80 % alcohol, again filtered and the residue washed with about 300 ml. of 80 % alcohol. The combined filtrates, concentrated *in vacuo* to about one-fourth of their original volume, were then poured on to 300 g. of corn starch and dried at

room temperature under an electric fan. Thus 300 g. of the final prepared starch contained the extract from 800 g. of the original cereal. In the preparation of the extract, allowance was made for the water content of the cereals, the first extraction usually being made with 85% alcohol. Diets were prepared, of which 100 g. contained the extract from 50 g. cereal (as in the original specification of Bourquin and Sherman) and from 150 g. of cereal, respectively. These diets may then be stated to contain 50 and 150 "gram equivalents" of cereal %, respectively. For convenience, they are called Diets B and S I and B and S III and their compositions are given below.

	Diet B and S I	Diet B and S III
Caseinogen	18	18
Salt mixture	4	4
Hardened cottonseed oil	8	8
Lard	2	2
Corn starch	49	12
Corn starch + cereal extract	19	56

Young rats which had been partially deprived of vitamin B₂ from birth, as described by Chick *et al.* [1935], were weaned at 21 days, when they weighed 30–40 g., placed in separate cages and given the above diets containing cereal extracts, together with vitamins A and D as a separate dose of cod-liver oil. Rats receiving the B and S I diets were given additional vitamin B₁ as Peters's concentrate. Otherwise, when they ate very little, it was feared that they might be short of this vitamin. It was assumed that rats receiving B and S III diets obtained enough vitamin B₁ except when the diets contained extracts of highly milled cereal products: in this case a supplement of vitamin B₁ was given.

INVESTIGATION OF ALCOHOLIC EXTRACTS OF CEREALS FOR THE PRESENCE OF VITAMIN B₆ AND FLAVIN.

(a) Incidence and cure of skin symptoms in rats receiving diets containing wheat extract.

In an initial experiment to investigate the alcoholic extract of wheat, a litter of 9 young rats was used: 5 rats received the B and S I, and 4 the B and S III type of diet. After 3–5 weeks all the animals showed a severe degree of the (b) type of generalised skin affection, which has been shown to be developed in the absence of flavin when vitamin B₆ is provided.

Of the 5 rats receiving the B and S I diet, 3 which had ceased to grow and had developed severe skin symptoms were dosed with hepaflavin (12 γ) daily for 4 weeks, during which period their skin lesions were healed and there was an average gain in weight of 8 g. weekly. One of the other two rats on this diet was given a dose of vitamin B₆ in the form of a yeast extract from which the vitamin B₁ and flavin had been removed by autoclaving and treating with fuller's earth [see Chick *et al.*, 1935], but this supplement caused no improvement of the severe (b) type of skin symptoms, nor was growth restored until flavin was given as well. The condition then improved rapidly and the rat gained an average of 18 g. weekly for 4 weeks on the combined dose. The fifth animal was in a very poor condition and died a few days after it was given a dose of flavin, without showing any remission of its symptoms.

The 4 rats which received the B and S III diet remained in a better condition than those receiving the B and S I diet and even grew slightly for 2 or 3 weeks. They also developed severe (b) type of skin symptoms after 6–10 weeks and 3 were given daily doses of 12 γ flavin. Their average weekly gain in weight was 18 g. for 4 weeks and their condition was good at the end of the experiment. The fourth animal of this group received a dose of vitamin B₆ for 3 weeks, during which it

showed no improvement and its condition became very bad. Improvement took place after an additional dose of 12 γ of flavin was given, but growth was sub-normal (average of 10 g. weekly) and the skin symptoms did not clear so quickly as those of other rats which had been deprived of flavin for shorter periods.

This supplementary effect of flavin added to a diet containing wheat extract, in curing the generalised skin condition developed on this diet, indicated that the alcoholic extract of whole wheat contained a fair amount of vitamin B₆ but was deficient in flavin.

(b) *Effect on growth of extracts from different cereals.*

With weight increase as criterion, a long series of experiments was made to study the vitamin B₂ components present in diets containing extracts of wheat, white top patent flour, maize, maize endosperm, ground polished rice. The diets were prepared to carry extracts supplying either 50 (B and S I) or 150 (B and S III) g. equivalents of whole cereal per 100 g. of diet, but for the milled cereal products, only diets carrying 150 g. equivalents were used, as it was thought probable that their content of vitamin B₆ would be much less than that of the corresponding whole grains.

Young rats given these diets at weaning sometimes grew slightly for 2 to 3 weeks, but their weight generally remained stationary at about 50 g. and they rapidly developed skin symptoms of the (b) type. Out of 42 rats which received these diets for 6 weeks, 37 showed the (b) type of symptoms. After 3-6 weeks the rats were dosed with flavin and the growth response to 12 γ daily of flavin for 4 weeks was observed. The skin symptoms cleared up in every case in which they were present. The results are summarised in Table I.

Table I.

Group	Cereal diet	No. of rats	Before flavin doses			After flavin doses		
			Food intake		Av. weekly increase in wt. g.	Food intake		Av. weekly increase in wt. g.
			g. per week*	g. equiv. of cereal per week*		g. per week	g. equiv. of cereal per week†	
1	B and S I wheat extract	7 (a)	38	19	2	30	15 (11-18)	7
		5 (b)				52	26 (21-32)	13
2	B and S III wheat extract	4 (a)	52	78	2.5	46	69 (55-78)	16
		3 (b)				60	91 (88-93)	19.5
3	B and S I maize extract	5 (a)	56	28	1.5	30	15 (13-19)	9
		6 (b)				52	26 (22-33)	13.5
4	B and S III maize extract	2 (a)	56	84	2.5	49	74 (69-77)	16
		7 (b)				58	89 (81-99)	15
5	B and S III white flour extract	8	36	54	2	52	77	11
6	B and S III polenta extract	11	29	44	1.5	50	75	11
7	B and S III ground rice extract	8	35	52	2	52	77	10

* The diets were fed as dry powders and at first there was some scattering so that the figures for intake in cols. 4 and 5 are to be regarded as approximate.

† Figures in brackets indicate the extent of variation within the group.

The variation in the intake of supplementary factors, vitamins B₁ and B₆, with variations in appetite, constitutes a fundamental difficulty in the use of the Bourquin and Sherman type of diet, but in these experiments the effect of any

variability in the amount of vitamin B₁ can be neglected, since the adequacy of the supply was assured by giving additional doses of Peters's vitamin B₁ concentrate from yeast in all cases where it was considered that the intake in the diet was low. The food intake was completely recorded in all experiments so that the amounts of vitamin B₆ ingested could also be checked.

It was found that intake and growth response varied considerably in individual rats, similarly treated, without relation to sex, so that the results were analysed on the basis of a division into groups (a) and (b) eating more or less than 20 g. equivalents weekly in the group receiving the B and S I type of diet, and more or less than 80 g. equivalents in the group receiving the B and S III diet. These amounts indicate not only the cereal extract consumed (and the vitamin B₆ consumption) but, in any one group, are also proportional to the calorie intake.

In the period before the diets were supplemented with flavin all the rats showed a uniform lack of growth whatever the intake of cereal extract; after flavin was given those rats receiving the B and S III diets showed much more satisfactory weight increases than those on B and S I diets. At the lower levels of intake there would not appear to be a great difference in effective supplementary factors in the extracts from wheat and maize, but at higher levels of intake wheat extract appears to be rather better than maize in growth-promoting effect. With wheat extract, increased intake in group 2b (Table I) is reflected in an increase in average growth rate, in group 4b with maize extract there is no such effect, and the possibility is suggested that the larger intake of the alcoholic extract of maize may even have had a slight depressing effect on growth.

Extracts from milled cereal products, when supplemented with flavin, elicited a much lower growth response in proportion to intake than the extracts from whole cereals. There is a striking agreement between the figures for intake and gain in weight in groups 5, 6 and 7, Table I, which indicates that the endosperm of these cereals contains a fairly constant, if somewhat low, amount of vitamin B₆. The average intake of the cereal extracts in these groups was about the same as that of the (a) groups receiving the extracts of whole wheat and maize in similar B and S III diets, but the growth response was much less. It must therefore be concluded that a considerable proportion of the vitamin B₆ present in the original cereal had been lost in milling and was therefore situated in the germ and integuments.

In some experiments the addition of extra vitamin B₆, in the form of a yeast extract from which vitamin B₁ and flavin had been removed, was investigated, but such addition did not usually improve the condition of the rats or increase the growth rate. This was to be expected as they were already receiving vitamin B₆ in the cereal extracts but were deficient in flavin.

It may be concluded from these experiments that the alcoholic cereal extracts are rich in vitamin B₆ and deficient in flavin. The latter observation is not unexpected as, even if flavin were present in the whole cereal, it would not easily be extracted by cold 80% alcohol.

Comparison of whole cereals and alcoholic cereal extracts as regards content of vitamin B₆ and flavin.

Birch and György [1935] found that only part of the vitamin B₆ activity of wheat and maize was extracted by cold 80% alcohol and the following tests with diets containing whole cereals and cereal extracts have confirmed this observation.

Diets containing 50 g. of whole wheat or maize per 100 g. of diet were compared with B and S I diets containing 50 g. equivalents of whole cereal per 100 g. of diet. A diet with white top patent flour was also made up to compare with the diet containing extract of flour (Table I). The composition of the diets, allowing for variations in the content of protein and carbohydrate in the cereal was as follows: caseinogen 11, salt mixture (McCollum's No. 185) 4, hardened cottonseed oil 7, lard 2, corn starch 26, cereal 50.

Exp. 1. These diets were tested on 25 young rats from 3 litters, groups of 5 receiving 50 % whole wheat diet, B and S I wheat extract diet, 50 % whole maize diet, B and S I maize extract diet and 50 % whole flour diet, respectively (see Table II).

Table II.

Group	Cereal diet	Before flavin doses				After flavin doses			
		No. of rats	Time in weeks	Weekly intake of cereal, g. or g. equiv.	Av. weekly increase in wt. g.	No. of rats	Time in weeks	Weekly intake of cereal, g. or g. equiv.	Av. weekly increase in wt. g.
Exp. 1:									
1	Whole wheat 50%	4	4	32	13.4	1	4	43	20
2	White flour 50%	5	4	14	1.0	5	4	21.9	10
3	Whole maize 50%	5	1	22	6.0	4	4	33	16.4
4	B and S I wheat extract	5	4	13	-0.3	4	4	20.4	9.0
5	B and S I maize extract	5	4	13	0.2	5	4	20	9.0
Exp. 2:									
6	Whole wheat 30%	4	2-4	7.5	7.2	1	4	22	22
7	Whole maize 30%	4	2-4	11	6.1	4	4	21	16.5
8	B and S I wheat extract	4	2-4	15	4.8	1	4	26	12.7
9	B and S I maize extract	4	2-4	16	4.6	1	4	28	14.5

The rats receiving the diet containing 50 % whole wheat grew steadily at a rate of 10-19 g. weekly without additional flavin, except in the case of 1 rat which ate very little in the first 2 weeks of the experiment and did not thrive until it received a dose of flavin, after which it grew 81 g. in 4 weeks. The 5 rats receiving the diet containing 50 % whole maize showed a much smaller increase in weight, only about 6 g. weekly, until they received additional flavin, when their growth rate was of the same order as that of their litter-mates receiving the whole wheat diet without added flavin. The results were complicated by differing food consumption, since the rats receiving the 50 % whole wheat diet ate 23-45 g. wheat per week, and those receiving the 50 % whole maize diet ate only 16-29 g. of maize per week before receiving the flavin doses but 20-40 g. afterwards.

The rats in groups 4 and 5 receiving diets containing 50 g. equivalents per 100 g. of wheat and maize as extract were all given flavin doses at the same time as litter-mates in group 3 receiving the 50 % whole maize diet, and their average growth was much less than that of the rats receiving the unsupplemented whole wheat diet, or those receiving the whole maize diet with additional flavin. Those receiving the wheat extract diet supplemented with flavin had an average intake of 20 g. equivalents of wheat weekly and only showed an average gain of 9 g. weekly, and those receiving the maize extract diet showed similar figures for both intake and growth. The amounts of extract consumed in groups 4 and 5 were not equivalent to the amounts of whole cereal consumed by rats in groups 1 and 3 receiving the whole cereal diets, but even so the growth response was so much less as to suggest that by no means all the vitamin B₆ of wheat and maize is extracted by 80 % alcohol at room temperature.

The results of this experiment did not give a quantitative estimate of the extractibility of the vitamin B₆ in wheat and maize by cold 80 % alcohol, but did

serve to demonstrate a notable difference between whole wheat and whole maize for the maintenance of growth and good condition and indicated the presence of the whole vitamin B₂ complex in whole wheat, but a lack of the flavin component in whole maize.

Rats receiving the diet containing 50% white flour, before they were given flavin, grew rather less than those receiving the whole maize diet and very much less than those receiving the whole wheat diet: their intake was, however, very low. After they were given doses of flavin their average weekly growth was 10 g. and average intake 22 g. of flour weekly. A comparison of these figures with those for group 5 in Table I shows a very similar growth result (weekly average 11 g.) for rats which ate 77 g. equivalents of flour as alcoholic extract, thus indicating that the alcoholic extract contained only one-third to one-quarter of the vitamin B₆ present in the flour.

Exp. 2. An experiment was made with diets containing 30% whole wheat and maize, in the hope of obtaining a better estimate of the vitamin B₆ content of these grains and of the extent to which it was extracted by alcohol. The results given in Table II show that in the case of wheat supplemented with flavin, with comparable intake of whole cereal and of g. equivalents of cereal extract (22–26 g. or g. equiv.), the growth with the former (weekly average 22 g.) far exceeds that with the latter (13 g.). A comparison of these figures with those of groups 1 (b) and 2 (b) in Table I suggests that such a difference in growth response corresponds to about three times the intake of cereal as g. equivalents, and if this calculation be permitted, it may be estimated that rather less than one-third of the vitamin B₆ in whole wheat is extracted by cold 80% alcohol.

The maize extract contained definitely less vitamin B₆ than an equivalent amount of whole maize, but no exact calculation of the proportion extracted could be made as there was no consistent increase in growth effect corresponding to increased intake either of the whole cereal or of the extract.

Further, this experiment provides more evidence of the fact that wheat contains more flavin than maize.

CONCLUSIONS.

These results confirm the observation of Birch and György [1935] that cereals are a good source of vitamin B₆. Wheat and maize seem to be about equally potent sources at levels of intake up to about 20–30 g. or g. equiv. of cereal weekly. At higher levels of intake there is evidence that maize was either a less effective source of vitamin B₆, or that its activity was prejudiced by the presence of some other factor, possibly harmful to rats. For whilst increased intake of wheat, either as such or as the 80% alcoholic extract, was accompanied by increased growth, a similarly increased intake of maize only allowed increased growth up to a limited amount, beyond which there was no further increase and sometimes even a slight falling off. Similar results were obtained with all types of maize diet used, whether containing whole maize or the extract (see Table I, groups 3 and 4: Table II, groups 3 and 7), so that if there is some deleterious factor in maize it would seem to be extracted by cold 80% alcohol.

The milled products of wheat and maize, and also ground polished rice, were found to be less active sources of vitamin B₆ than the whole cereals. A comparison of groups 5 and 6 in Table I with groups 1 and 3, respectively, suggests that flour and polenta contain only about one-third of the amount of vitamin B₆ present in the whole grains, since when supplemented with flavin about three times the intake (calculated as g. equiv.) is needed to support a similar growth rate. In

the case of flour this result agrees with that derived from a comparison of the unextracted materials, which showed that white flour was much inferior to whole wheat as a source of vitamin B₆. It is therefore apparent that much of the vitamin B₆ of the grain is contained in the germ and integuments.

Whole wheat was also found to be a slightly better source of the entire vitamin B₆ complex than whole maize, but when the whole maize diets were supplemented with flavin the growth rate became approximately equal to that obtained with whole wheat unsupplemented. Whole wheat would therefore appear to contain a greater amount of flavin than whole maize. This conclusion has been confirmed by Dr Ellinger (private communication) by chemical work, in which an attempt was made at quantitative extraction and estimation of flavin in wheat and maize.

The experiments made with the Bourquin and Sherman diet indicated that the flavin of cereals was not extracted with cold 80% alcohol, but that about one-third to one-quarter of the vitamin B₆ could be removed by two extractions with cold 80% alcohol. This finding is not in accord with that of Birch *et al.* [1935, pp. 2836, 2849] who found this vitamin to be extracted with great difficulty.

A significant nutritional difference has thus been demonstrated between wheat and maize in the larger content of flavin in wheat. The experiments in this paper permit no other conclusion. The result is, however, opposed to that of Birch *et al.* [1935] who found flavin present in maize in greater amount than in wheat. We can suggest no explanation for this discrepancy unless there is considerable variation in the flavin content of different samples of maize.

It is important to note that the use of the Bourquin and Sherman type of diet in investigations of this type is not very satisfactory, since the variation in intake of food with the appetite of the experimental animals also causes variation in the intake of certain essential dietary factors and makes results more difficult of interpretation. A more reliable result would be obtained by giving the test materials as separate doses, as in a few experiments reported elsewhere [Copping, 1936].

PELLAGRA AND MAIZE-EATING.

It would be tempting to emphasise the significance of the deficiency of flavin in maize, seeing that many substances which prevent and cure pellagra (liver, liver extract, meat, milk, yeast) are rich in flavin. Clinical trials have not, however, indicated any curative property for human pellagra in pure flavin [Dann *et al.*, 1935; Spies and Chinn¹] nor can black tongue in dogs be cured by it [György, 1935]. Further, white flour, on which so many wheat eaters subsist, is equally deficient in flavin and populations living in poverty in isolated districts and subsisting too exclusively on milled wheaten products tend to develop beriberi, whilst endemic pellagra is unknown in such circumstances [Aykroyd, 1930].

There is, however, in some of the experiments recorded in this paper a suggestion of an unwholesome element in maize. In several tests, increasing the amounts of maize or maize extracts in the diet, even when these were providing an essential vitamin, did not show an increasing beneficial effect, as was the case in similar trials with wheat and wheat extracts. The theory postulating some toxic factor in maize and regarding endemic pellagra as a disease of intoxication has been put forward by many investigators, chiefly on clinical grounds, and is possibly supported by this observation.

¹ See Birch *et al.* [1935, p. 2850].

SUMMARY.

1. The results of experiments on rats with diets of the Bourquin and Sherman type, containing extracts of wheat or maize or their milled products, or diets containing the unextracted whole cereals, indicate that wheat and maize are good sources of vitamin B₆ and that one-third to one-quarter of the vitamin B₆ present is extracted by cold 80 % alcohol.

2. Wheat appears to contain a greater amount of flavin than maize, but no flavin is extracted by cold 80 % alcohol.

3. The vitamin B₆ in both wheat and maize appears to be present in greater amount in the germ and integuments than in the endosperm.

4. Some of the experiments with maize and maize extracts suggest the possibility that a deleterious substance is present in this cereal, and is, moreover, extracted by cold 80 % alcohol.

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CXXV. THE EFFECT OF INCOMPLETE DIETS ON THE CONCENTRATION OF ASCORBIC ACID IN THE ORGANS OF THE RAT.

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It has been known for some time from biological tests that the liver of animals contains vitamin C in very much higher concentrations than many of their other tissues. Recent observations have brought to light that the small intestine also is rich in the vitamin. Harde and Wolff [1934] were the first to demonstrate that, of the entire intestinal tract, the small intestine of the mouse kept on a scorbutic diet showed by the indophenol titration method a more or less constant ascorbic acid content about equal to that of the liver—an observation which led them to suggest that the vitamin was most probably synthesised by the former. A few months later Hopkins [1934] made a similar observation with regard to the rat and further extended the investigation in order to determine its connection with the vitamin content of the liver and the relation of the concentrations in these organs to the composition of the diet consumed by the rat. Soon after the publication of the above communication the writer [Zilva, 1935] produced evidence which militated against the view that the concentration of ascorbic acid in the small intestine of the mouse or rat was due to the synthesis of the vitamin in that organ. He was able to show that when guinea-pigs—animals which do not synthesise ascorbic acid—were kept on a good mixed diet containing cabbage *ad lib.* extracts of the walls of the small intestine possessed a reducing capacity for indophenol of an order similar to that of the mouse and the rat. When, however, the vitamin was withheld from the diet the reducing capacity fell almost to zero. Furthermore, when ascorbic acid was injected into the blood stream of vitamin C-depleted animals the reducing capacity for indophenol rose to about the level found in the small intestine of guinea-pigs subsisting on a diet rich in vitamin C. The results indicated that it was more probable that the mouse and the rat, like the guinea-pig, tend to concentrate and store rather than to synthesise ascorbic acid in the small intestine. From the writer's experiments with guinea-pigs it could be concluded in addition that the indophenol-reducing substance in the walls of the small intestine of the mouse and rat was mainly ascorbic acid. Similar results were also obtained independently by Jacobsen [1935].

In a recent publication Hopkins [1935]² with the assistance of B. R. Slater gives in greater detail the results and conclusions previously outlined in brief in his preliminary note [Hopkins, 1934]. He found that when rats existed on a

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² In discussing the origin of ascorbic acid in connection with animals which synthesise the vitamin Hopkins says in this communication "That it (ascorbic acid) is normally present in the latter (the small intestine) is now well known [Hopkins, 1934; Harde and Wolff, 1934; Zilva, 1935; *et al.*]". For the sake of precision the writer would like to point out that in his publication here referred to he demonstrated the presence of ascorbic acid in the intestine of the guinea-pig and not of the rat or mouse, the object being, as mentioned above, to stress the improbability of synthesis in the small intestine implied by Harde and Wolff and by Hopkins in their communications.

normal mixed diet the average concentrations of ascorbic acid in the liver and in the small intestine were the same. When however a carbohydrate diet was consumed by these animals the concentration in the liver rose immediately, becoming higher than in the intestine. On the other hand on diets lacking carbohydrates such as those consisting of protein and fat or fat alone and after 48 hours' starvation the concentration in the liver rapidly fell while that in the intestine rose. He therefore considers that the liver and the intestine represent sites of formation rather than of storage of the vitamin. When carbohydrate in the liver is inadequately available as a primary source for synthesis, the intestine can then utilise protein for this purpose. A mutual adjustment is thus possible in the productive activities of these organs.

This work appeared to call for repetition. From the data provided in this communication it will be seen that some of the major results could not be reproduced, whilst others when considered in their entirety are subject to an interpretation different from that given to them by Hopkins.

Technique.

Although the technique used by Hopkins was in principle the same as is usually applied to work of this nature, all the conditions were adjusted as nearly as possible to those observed by him in his work.

Stock. Rats raised in two different places, namely the Lister Institute and by a breeder in Hampshire were employed. The origin of these experimental animals was found, however, not to influence the results. Each experimental animal was kept in a separate cage.

Diets. Rats on the normal diet were offered a free choice of brown bread, milk, oats and bran. The carbohydrate diet contained three parts of starch and one part of cane sugar; the protein and fat diet two parts of caseinogen and one part of lard. The last two diets were made into a paste, the first with about one-quarter part by weight of water, the second with about an equal part of water. The fat diet consisted of lard only. To each of the diets about 5% of a balanced salt mixture was added. Clean water changed daily was offered *ad lib.* in all cases during the experiment, including the 48-hour fasting period. The amount of food consumed was recorded daily.

Preparation and extraction of tissues. All the animals were killed by stunning and bleeding. The livers and the intestines were taken out immediately, and after removal of all adhering tissues were worked up for analysis with the utmost speed. The intestines extending from the pyloric to the caecal end were emptied of their contents by a flow of tepid water, squeezed and well dried on filter-paper. The organs were then thoroughly ground with sand in three times their weight of dilute trichloroacetic acid, so as to yield a final extract containing 2.5% trichloroacetic acid.

Titrations. All the titrations were carried out by running the indicator into 5 ml. and occasionally less of the colourless extracts adjusted to p_H 2.5 from a macro-burette, until the addition of two drops left the solution coloured for 10 sec. Each extract was titrated with both dimethylaminobenzindophenol and 2:6-dichlorophenolindophenol. These figures usually agreed very well. When there was a small difference the average of the two titrations was used in the calculation of the result, the average dry weight of the organs being taken as a basis. In a number of representative experiments the extracts were also titrated with both indophenols firstly by delivering the indicators from a micro-burette into the extracts and secondly by running the extracts from such a burette into a measured quantity of 2:6-dichlorophenolindophenol of known strength

(0.2 ml. *N*/200 or 0.05 ml. *N*/100). The control micro-determinations were carried out independently by Mr A. E. Kellie to whom I should like to express my thanks for assistance in this matter. The indicators were standardised daily against pure ascorbic acid.

Determination of dry matter contents. The adhering fatty and other tissues were carefully removed from the organs. They were then dried at 37° for about 24 hours, after which they were ground to a fine powder and dried to constant weight at 100°. All the intestines and some of the livers were extracted with ether in a Soxhlet apparatus during 1 working day previous to drying at 100°.

Discussion of results.

Before considering the general results reference must be made to the conditions under which the determinations of ascorbic acid were carried out. The writer and his colleagues have during the last 10 years been employing the indophenol technique with good results when this reduction method could be applied specifically in the assessment of the vitamin. The standardised indicator, phenolindophenol or dimethylaminobenzoindophenol used by them was always delivered into about 5 ml. of test solution until the addition of a few drops left the solution coloured for a specified time. Later Tillmans, using the somewhat more highly coloured 2:6-dichlorophenolindophenol which, as well as the above indophenols, is decolorised by ascorbic acid and compounds of a similar or greater reducing potential, also obtained good results by titrating in the same manner. Still later Szent-Györgyi made the useful suggestion of titrating in acid solution in order to suppress the reducing activity of some accompanying substances [*cf.* Harris and Ray, 1933]. This was followed by the introduction by numerous workers of minor modifications the utility of which is not immediately obvious. One of these [Birch *et al.*, 1933] consisted of delivering the test solution into the indicator instead of the reverse way of titration. Hopkins utilised this variant of indophenol titration and found it an excellent method. In view of the lack of agreement between some of the results in the two investigations it was necessary to ascertain whether it could be traced to the slight difference in the titration procedure. The results obtained (Table I) by the various procedures show that such differences as were observed could not be traced to one particular mode of titration but were due to a general error inherent in the indophenol technique as applied to animal tissue extracts. It may therefore be concluded that the divergencies in the results are not due to the minor variation in the technique and that the inverted order of titration with 2:6-dichlorophenolindophenol, apart from the personal predilection, does not, when considered objectively, endow the results with any higher degree of accuracy.

In this investigation not only are data given of the concentrations of ascorbic acid in the intestine and liver of the rats kept on the various diets but also of the total contents of the vitamin in these organs together with their weight. These latter data were not provided or considered by Hopkins, but, as will be seen later, they form evidence of an essential character in the argument of the thesis.

For the sake of convenience the average results of all the experiments are given in Table VIII, p. 867.

The first experiment to be considered will be that of the rats kept on the stock diet, since the results obtained with them must serve as the standard for comparison. Considering that these rats had a free choice of a mixed diet it was difficult to record the quantity of food consumed by them per day but from various observations it may be estimated to be about 10–15 g. calculated on dry matter. In this and in all the other experiments, the results are divided into

Table I.

Liver					Intestine				
Macro direct Dim. mg./g.	Macro direct Dichlor. mg./g.	Micro direct Dim. mg./g.	Micro direct Dichlor. mg./g.	Micro inverted Dichlor. mg./g.	Macro direct Dim. mg./g.	Macro direct Dichlor. mg./g.	Micro direct Dim. mg./g.	Micro direct Dichlor. mg./g.	Micro inverted Dichlor. mg./g.
48 hours without food.									
0.29	0.30	0.30	0.29	0.31	0.16	0.16	0.18	0.17	0.20
0.21	0.20	0.22	0.20	0.21	0.18	0.17	0.20	0.19	0.20
0.23	0.22	0.22	0.22	0.23	0.27	0.27	0.28	0.26	0.30
0.23	0.21	0.26	0.24	0.23	0.23	0.23	0.27	0.24	0.28
0.20	0.21	0.22	0.20	0.20	0.21	0.20	0.23	0.23	0.24
0.31	0.33	0.33	0.29	0.33	0.31	0.30	0.28	0.25	0.28
0.30	0.29	0.29	0.28	0.30	0.27	0.27	0.28	0.28	0.29
0.15	0.17	0.17	0.18	0.16	0.32	0.40	0.34	0.35	0.34
0.18	0.18	0.16	0.16	0.16	0.30	0.31	0.30	0.29	0.31
0.28	0.30	0.30	0.31	0.34	0.31	0.30	0.28	0.28	0.24
Av.	0.24	0.24	0.25	0.24	0.25	0.26	0.26	0.26	0.27
3 days on carbohydrate diet after 2 days' fast.									
0.20	0.20	0.17	0.17	0.23	0.10	0.10	0.10	0.10	0.14
0.29	0.29	0.26	0.27	0.33	0.14	0.16	0.18	0.17	0.17
0.35	0.35	0.30	0.30	0.34	—	0.16	0.16	0.15	0.18
0.34	0.34	0.30	0.30	0.31	0.16	0.16	0.15	0.14	0.16
0.24	0.24	0.24	0.23	0.25	0.10	0.10	0.12	0.10	0.11
0.36	0.36	0.35	0.34	0.37	0.10	0.12	0.13	0.13	0.14
0.22	0.22	0.21	0.21	0.22	0.15	0.15	0.18	0.17	0.18
0.29	0.29	0.29	0.28	0.28	0.18	0.18	0.19	0.19	0.20
Av.	0.29	0.29	0.27	0.26	0.29	0.13	0.14	0.15	0.16
3 days on protein-fat diet after 2 days' fast.									
0.31	0.32	0.36	0.35	0.36	0.35	0.35	0.31	0.31	0.32
0.16	0.17	0.14	0.15	0.18	0.24	0.23	0.24	0.21	0.25
0.21	0.19	0.20	0.18	0.23	0.21	0.19	0.20	0.20	0.23
Av.	0.23	0.23	0.23	0.26	0.27	0.26	0.25	0.24	0.27

Dim. = dimethylaminobenzoinodophenol. Dichlor. = 2:6-dichlorophenolindophenol.

two sections—one consisting of those obtained with animals weighing between 150 and 200 g., such as are most of those used by Hopkins, and the other of those of heavier rats. It is seen (Table II) that as the animals grew the total amount of ascorbic acid in both organs increased but the concentrations remained more or less the same. In comparing the respective results of the two communications it was found that whilst the average concentration observed in the livers was essentially the same in both cases, that of the intestines as recorded by Hopkins (0.26 mg./g.) is rather higher than the figure found in the present investigation (0.19 mg./g.).

The results next to be considered are those obtained with the animals receiving no food for 48 hours (Table III). Their livers showed the same concentration as that found in the case of the rats on the stock diet. On the other hand the intestines of the fasted animals belonging to the 150–200 g. group showed a rise of possible significance in the vitamin concentration as compared with the same group on the stock diet. This difference is similar to but of a lower order than that found by Hopkins (0.26 mg./g. for stock and 0.40 mg./g. for fasted rats). The latter suggested that the increase in the concentration in the gut was due to synthesis of the vitamin in the organ owing to “a specific effect of failure in carbohydrate supply”. The present writer's figures offer a different interpretation from the above. A fast of 48 hours' duration, as would naturally

Table II.

Stock diet.								
Wt. of rat g.	Sex	Wet wt. of liver g.	Ascorbic acid liver		Wet wt. of intestine g.	Ascorbic acid intestine		
			mg./g.	Total content mg.		mg./g.	Total content mg.	
150	C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃	5.5	0.25	1.4	6.0	0.21	1.3	
152		5.5	0.22	1.2	6.0	0.20	1.2	
152		5.5	0.24	1.3	6.0	0.18	1.1	
157		6.0	0.23	1.4	6.0	0.16	1.0	
172		5.0	0.24	1.2	7.0	0.19	1.3	
175		5.5	0.22	1.2	7.0	0.17	1.2	
195		7.0	0.19	1.3	7.5	0.20	1.5	
160		6.0	0.27	1.6	6.0	0.18	1.1	
155		5.0	0.26	1.3	5.0	0.26	1.3	
160		5.5	0.27	1.5	6.0	0.20	1.2	
135	C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃	4.0	0.23	0.9	6.0	0.20	1.2	
155		5.0	0.36	1.8	6.0	0.25	1.5	
140		5.5	0.31	1.7	7.0	0.17	1.2	
197		6.5	0.26	1.7	7.5	0.17	1.3	
180		6.0	0.30	1.8	8.0	0.19	1.5	
170		5.0	0.26	1.3	8.0	0.14	1.1	
Av.		163	5.5	0.26	1.4	6.6	0.19	1.3
200		C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃ C ₃	7.0	0.21	1.5	9.0	0.19	1.7
210			5.5	0.27	1.6	5.5	0.32	1.8
*255			11.0	0.22	2.4	11.0	0.25	2.8
265	10.0		0.24	2.4	5.5	0.20	1.1	
295	11.0		0.31	3.4	11.0	0.16	1.8	
335	13.0		0.20	2.6	10.0	0.19	1.9	
410	13.0		0.28	3.6	9.0	0.19	1.7	
230	8.0		0.34	2.7	6.5	0.29	1.9	
270	10.0		0.32	3.2	9.0	0.21	1.9	
Av.	274		9.8	0.27	2.6	8.5	0.22	1.8

* Pregnant.

be expected, produced a loss in the dry matter including a large part of the ether-extractable fraction (Table IV) accompanied by a corresponding fall in the wet weight of the intestine, and, as the total vitamin content of this organ remained unaltered, the concentration per g. of tissue in the lighter group of animals (such as used by Hopkins), therefore, rose in consequence. It is essential in interpreting results such as these that the weight of the organ and its total content of ascorbic acid and not the concentration alone be taken into account—a consideration omitted by Hopkins from his argument. It may be further noted in this connection that there was a similar diminution in the weights of the livers but in this case it was accompanied by a corresponding diminution in the total vitamin content of these organs. This observation however has no direct connection with the present argument. Further work will be required to ascertain whether the decrease is significant.

The experiment which has the strongest bearing on the thesis under discussion is that carried out on rats receiving a carbohydrate diet. The results of the present investigation are given in Table V. The first point that emerges from them is that the incomplete diet had a detrimental effect on the animals. This is reflected in their appreciable loss of weight. Furthermore when the figures for the dry matter content and for the ether-extractable fraction of the intestine are examined (Table IV) a condition similar to but not quite so marked as that found in the case of the fasted rats is observed, namely a loss in weight in each of the fractions as compared with the corresponding figures recorded in the analysis of

Table III.
48 hours without food.

Wt. of rat		Sex	Wet wt. of liver	Ascorbic acid liver		Wet wt. of intestine	Ascorbic acid intestine	
Initial g.	Final g.			mg./g.	Total content mg.		mg./g.	Total content mg.
140	110	O ₃	4.0	0.15	0.6	4.0	0.32	1.3
210	195		6.0	0.28	1.7	6.0	0.31	1.9
185	160		5.0	0.18	0.9	5.0	0.30	1.5
130	115	O ₃ + O ₂	3.2	0.41	1.3	4.5	0.27	1.2
135	115		3.5	0.34	1.2	4.5	0.29	1.3
175	145		4.3	0.26	1.1	6.0	0.21	1.3
180	160	O ₃ + O ₂ + C ₁	4.5	0.36	1.6	5.3	0.26	1.4
185	162		5.4	0.31	1.7	5.6	0.21	1.2
195	170		5.0	0.28	1.4	4.5	0.36	1.6
145	120	O ₃ + O ₂ + O ₃	4.0	0.18	0.7	6.0	0.22	1.3
210	185		5.5	0.24	1.3	5.5	0.25	1.4
215	192		5.5	0.33	1.8	6.0	0.28	1.7
150	132	O ₃ + O ₂ + O ₃ + O ₂	4.5	0.13	0.6	7.0	0.19	1.3
195	172		5.0	0.28	1.4	6.0	0.25	1.5
205	185		5.0	0.22	1.1	7.5	0.19	1.4
Av.	177	155	4.7	0.26	1.2	5.6	0.26	1.4
280	250	O ₃ + O ₂ + O ₃ + O ₂ + C ₁	7.0	0.29	2.0	7.0	0.16	1.1
300	270		8.0	0.21	1.7	8.5	0.18	1.5
285	252		6.0	0.23	1.4	7.0	0.27	1.9
410	380	O ₃ + O ₂ + O ₃ + O ₂ + C ₁	9.0	0.23	2.1	6.0	0.23	1.4
375	347		8.0	0.20	1.6	7.0	0.21	1.5
295	280		7.0	0.31	2.2	6.0	0.31	1.9
280	240	O ₃ + O ₂ + O ₃ + O ₂ + C ₁	7.0	0.27	1.9	8.0	0.29	2.3
310	260		8.0	0.30	2.4	6.0	0.27	1.6
280	252		6.5	0.25	1.6	7.0	0.17	1.2
360	332	O ₃ + O ₂ + O ₃ + O ₂ + C ₁	8.6	0.28	2.4	8.5	0.32	2.7
250	225		6.0	0.25	1.5	6.7	0.16	1.1
Av.	311	281	7.4	0.26	1.9	7.1	0.23	1.7

the intestines of the rats on the stock diet. 3 days on the incomplete diet therefore did not bring the condition of the animals up to the level observed in the stock rats. In the light group of the previously fasted animals there was some rise in the total ascorbic acid content of the liver. This increase was, however, not observed in the case of the heavier group and of the group which subsisted for 6 days on the diet without a previous fast. The vitamin concentrations of these 3 groups were 0.30, 0.31 and 0.26 mg./g. respectively as compared with 0.26 and 0.27 mg./g. the figures for the corresponding groups in the stock diet animals. It seems rather doubtful whether this rise of 15% in the concentration of the reducing substance extracted from the livers of the previously fasted animals is fully significant, especially as no increase in concentration, such as was found by Hopkins, could be recorded in the animals which had no previous fast. His figures were 0.35 mg./g. for the animals after 2 days' fast and 3 days on the carbohydrate diet and 0.32 mg./g. for those on 5-7 days of the diet without previous fast as compared with 0.26 mg./g. for the rats on the stock diet, thus registering an increase of 34.4 and 23% respectively, figures much higher than those above. Unlike his results there was also a greater variation in the liver vitamin concentrations of the individual animals in the present investigation. From Table V it is further seen that a significant fall is recorded in the average vitamin content and in the average vitamin concentration in the intestines of all the groups on the carbohydrate diet as compared with the stock diet group, in spite of the loss in the wet weight. This observation is also at variance with that

Table IV.

Wt. of rat		Sex	Wet wt. of liver g.	Total dry wt. g.	Wt. of ethereal extract g.	% Total dry wt. Wet wt.	Wet wt. of in- testine g.	Total dry wt. g.	Wt. of ethereal extract g.	% Total dry wt. Wet wt.
Initial g.	Final g.									
Stock diet.										
	150	♀	6.50	1.77	—	27.21	6.31	1.21	0.30	19.18
	135	♀	7.34	1.88	—	25.66	6.71	1.17	0.23	17.43
	150	♀	6.09	1.70	0.16	27.93	6.86	1.06	0.21	15.44
	155	♀	5.42	1.49	0.13	27.48	5.00	0.97	0.25	19.38
	250	♀	8.76	2.63	—	29.98	6.41	1.44	0.54	22.50
	250	♀	8.81	2.62	—	29.70	7.46	1.22	0.26	16.39
	290	♀	12.18	3.16	—	25.98	9.87	1.77	0.28	17.98
	145	♀	11.70	3.33	—	28.48	6.25	1.14	0.26	18.22
	152	♀	—	—	—	—	4.98	1.02	0.28	20.54
	150	♀	—	—	—	—	7.10	0.94	0.24	13.21
Av.	183		7.42	2.06	0.15	27.80	6.69	1.19	0.28	18.03
48 hours without food.										
	177	♀	3.87	1.07	0.09	27.65	5.01	0.60	0.09	12.07
	175	♀	3.97	1.11	0.11	28.08	4.29	0.67	0.12	15.63
	180	♀	4.56	1.28	—	28.14	5.23	0.77	0.10	14.82
	177	♀	4.02	1.10	—	27.43	4.62	0.69	0.12	14.84
	185	♀	4.48	1.27	—	28.33	5.75	0.76	0.11	13.30
	180	♀	4.10	1.20	—	29.33	4.75	0.68	0.10	14.32
	270	♀	5.90	1.88	—	31.81	4.80	0.87	0.19	18.17
	305	♀	7.55	2.36	—	31.28	5.98	1.10	0.21	18.37
*300	270	♀	7.28	2.35	—	32.31	5.82	0.81	0.10	13.85
	270	♀	6.31	1.99	—	31.46	6.92	1.02	0.26	14.73
	280	♀	6.83	2.18	—	31.85	7.48	1.14	0.20	15.26
	170	♀	—	—	—	—	5.95	0.75	0.09	12.52
	160	♀	—	—	—	—	5.14	0.62	0.15	12.13
*175	125	♀	—	—	—	—	4.60	0.58	0.07	12.49
Av.	215		5.35	1.62	0.10	29.79	5.45	0.79	0.14	14.46
3 days on carbohydrate diet after 2 days' fast.										
	200	♀	6.70	1.83	0.22	27.39	6.18	0.88	0.19	14.29
	200	♀	6.16	1.56	0.19	25.31	6.25	0.98	0.13	15.65
	215	♀	7.14	1.87	0.23	26.18	6.91	1.03	0.19	14.94
	170	♀	6.90	2.06	0.37	29.83	5.14	0.66	0.10	12.80
	165	♀	6.10	1.68	0.18	27.60	5.93	0.94	0.17	15.81
	180	♀	7.12	1.97	0.18	27.67	5.76	0.92	0.15	16.05
	180	♀	6.88	1.95	—	28.31	5.58	0.90	0.16	16.21
	180	♀	7.73	2.23	—	28.85	6.03	1.01	0.19	16.78
	180	♀	5.56	1.56	—	28.11	5.36	0.80	0.12	14.91
Av.	186		6.70	1.86	0.23	27.69	5.90	0.90	0.16	15.27
3 days on protein-fat diet after 2 days' fast.										
	150	♂	5.67	1.71	0.24	30.11	6.26	1.00	0.17	15.99
	140	♂	4.36	1.32	0.12	30.13	6.19	0.74	0.08	11.93
	150	♂	5.67	1.75	0.22	30.81	6.82	0.87	0.14	12.81
	172	♂	6.03	1.80	—	29.77	7.12	0.99	0.15	13.96
	180	♂	5.13	1.55	—	30.14	5.46	0.81	0.08	14.77
	160	♂	6.08	1.71	—	28.17	6.38	1.03	0.19	16.13
Av.	159		5.49	1.64	0.19	29.86	6.37	0.91	0.13	14.26
3 days on fat diet after 2 days' fast.										
	160	♀	3.72	1.19	0.28	31.88	4.64	0.73	0.14	15.83
	190	♀	7.37	2.04	0.43	27.72	9.90	1.61	0.34	16.27
	175	♀	5.81	1.63	0.45	28.07	7.58	1.35	0.37	17.84
	180	♀	5.60	1.84	0.69	32.90	6.38	1.09	0.28	17.12
Av.	176		5.63	1.68	0.46	30.14	7.13	1.20	0.28	16.77

* Pregnant.

Table V.

Wt. of rat		Sex	Food intake g.	Wet wt. of liver g.	Ascorbic acid liver		Wet wt. of in- testine g.	Ascorbic acid intestine		
Initial g.	Final g.				mg./g.	Total content mg.		mg./g.	Total content mg.	
3 days on carbohydrate diet after 2 days' fast.										
180	135	O ₃	5	3.8	0.29	1.1	5.5	0.15	0.8	
160	137		8	5.0	0.28	1.4	5.0	0.14	0.7	
160	140		9	6.5	0.28	1.8	5.0	0.18	0.9	
165	140		10	5.0	0.26	1.3	5.5	0.15	0.8	
175	150		11	5.5	0.26	1.4	5.5	0.11	0.6	
160	150		12	4.5	0.33	1.5	6.0	0.13	0.8	
170	152		13	5.5	0.37	2.0	6.0	0.15	0.9	
*177	155		12	5.0	0.38	1.9	5.5	0.17	0.9	
190	155		11	6.0	0.28	1.7	6.2	0.15	0.9	
190	165		10	6.2	0.23	1.4	7.5	0.10	0.8	
183	165	O ₃ + C ₃	11	5.8	0.24	1.4	5.5	0.15	0.8	
180	170		11	5.0	0.28	1.4	5.5	0.15	0.8	
190	170		10	6.0	0.32	1.9	6.0	0.21	1.3	
195	172		14	5.0	0.38	1.9	6.0	0.17	1.0	
190	175		7	6.5	0.34	2.2	6.0	0.18	1.1	
215	197		14	7.0	0.29	2.0	6.5	0.17	1.1	
200	190		14	10.0	0.28	2.8	5.7	0.16	0.9	
190	180		13	7.3	0.38	2.8	5.0	0.18	0.9	
185	172		11	7.5	0.36	2.7	6.0	0.13	0.8	
195	185		13	7.5	0.20	1.5	6.0	0.10	0.6	
180	165	O ₃ + C ₃ + H ₂ O	9	8.0	0.29	2.3	5.7	0.16	0.9	
175	145		7	5.5	0.35	1.9	5.0	0.16	0.8	
190	162		8	6.5	0.34	2.2	5.7	0.16	0.9	
195	170		8	7.0	0.24	1.7	5.0	0.10	0.5	
190	170		7	7.0	0.36	2.5	5.0	0.12	0.6	
175	142		6	6.0	0.22	1.3	6.0	0.15	0.9	
170	150		6	6.5	0.29	1.9	5.5	0.18	1.0	
Av.	186		161	10	6.2	0.30	1.9	5.7	0.15	0.9
215	200		O ₃ + C ₃ + H ₂ O	15	8.5	0.32	2.7	5.5	0.15	0.8
220	200			14	8.0	0.22	1.8	7.0	0.11	0.8
230	205	10		7.5	0.33	2.4	6.0	0.15	0.9	
215	205	13		7.0	0.33	2.3	5.5	0.16	0.9	
230	220	14		5.5	0.40	2.2	6.5	0.22	1.4	
210	220	12		6.5	0.24	1.5	5.5	0.13	0.7	
225	225	16		8.0	0.36	2.9	7.0	0.17	1.2	
230	237	13		8.5	0.25	2.1	7.0	0.11	0.8	
Av.	222	214		13	7.4	0.31	2.2	6.3	0.15	0.9
6 days on carbohydrate diet.										
190	185	O ₃ + C ₃ + H ₂ O	10	6.0	0.27	1.6	6.0	0.14	0.8	
185	190		13	6.0	0.37	2.2	6.0	0.20	1.2	
170	152		11	5.0	0.26	1.3	6.0	0.18	1.1	
167	140		9	5.0	0.24	1.2	5.0	0.14	0.7	
170	150		13	4.0	0.30	1.2	5.5	0.16	0.9	
162	142		10	4.0	0.25	1.0	5.0	0.20	1.0	
160	150		8	5.5	0.22	1.2	5.0	0.14	0.7	
170	150		8	5.0	0.18	0.9	6.5	0.09	0.6	
150	137		7	4.5	0.18	0.8	5.0	0.12	0.6	
260	247		10	7.5	0.33	2.5	6.5	0.18	1.2	
270	265	O ₃ + C ₃ + H ₂ O	13	9.0	0.27	2.4	7.0	0.19	1.3	
Av.	187		163	10	5.6	0.26	1.5	5.8	0.16	0.9

Pregnant.

made by him since he found that the intestinal concentration of the vitamin was unaffected by the carbohydrate diet. A point of particular interest recorded here is that the total vitamin content of the intestines of the heavier animals instead of being proportionately greater than that of the corresponding lighter animals, as was the case in both the preceding experiments and in the livers of the animals of this experiment, was actually only equal to it. Thus in effect the fall in the case of the larger animals was even much more marked than in the smaller animals. It is therefore seen from the above data that the crucial evidence of Hopkins's hypothesis cannot be easily reproduced, a fact which, even without questioning the identity of the substance responsible for the increased reduction, dis-

Table VI.

Wt. of rat		Sex	Food intake g.	Wet wt. of liver g.	Ascorbic acid liver		Wet wt. of in- testine g.	Ascorbic acid intestine		
Initial g.	Final g.				mg./g.	Total content mg.		mg./g.	Total content mg.	
3 days on protein-fat diet after 2 days' fast.										
	177	155	♀	8	5.0	0.16	0.8	7.0	0.23	1.6
	177	150		7	5.3	0.17	0.9	8.0	0.18	1.4
	180	157		8	5.5	0.35	1.9	7.0	0.31	2.2
	*180	170		9	8.5	0.20	1.7	7.4	0.20	1.5
	180	150		8	5.7	0.18	1.0	6.5	0.18	1.2
	*182	155		7	6.5	0.26	1.7	7.0	0.27	1.9
	185	160		8	6.5	0.26	1.7	7.0	0.24	1.7
	200	180		7	8.0	0.25	2.0	8.0	0.25	2.0
	220	200		9	6.5	0.29	1.9	6.0	0.30	1.8
	200	200		8	8.7	0.17	1.5	8.0	0.26	2.1
	180	162	♂	7	7.0	0.19	1.3	7.0	0.29	2.0
	175	155		5	6.0	0.23	1.4	5.0	0.26	1.3
	220	192		9	6.0	0.20	1.2	10.0	0.22	2.2
	220	192		7	6.5	0.20	1.3	9.0	0.24	2.2
Av.	191	170		8	6.6	0.22	1.5	7.4	0.25	1.8
	260	225	♀	9	9.0	0.27	2.4	9.0	0.21	1.9
	280	262		10	10.0	0.24	2.4	11.0	0.17	1.9
	240	230		8	7.5	0.21	1.6	8.0	0.15	1.2
	290	270		9	10.0	0.20	2.0	10.0	0.16	1.6
	285	270		9	8.5	0.21	1.8	9.0	0.17	1.5
Av.	271	251	9	9.0	0.23	2.0	9.4	0.17	1.6	
5 days on protein-fat diet.										
	140	127	♀	9	5.2	0.19	1.0	6.0	0.20	1.2
	142	135		5	5.0	0.18	0.9	6.0	0.22	1.3
	142	130		5	5.5	0.22	1.2	6.0	0.23	1.4
	150	115		4	8.5	0.21	1.8	9.5	0.23	2.2
	160	150		6	5.0	0.18	0.9	6.2	0.15	0.9
	210	197		7	8.0	0.25	2.0	6.7	0.28	1.9
	215	175		3	6.0	0.18	1.1	6.0	0.15	0.9
	215	180		5	7.0	0.27	1.9	7.0	0.20	1.4
Av.	172	151		6	6.3	0.21	1.3	6.7	0.21	1.4
12 days on protein-fat diet.										
	170	137	♀	5	5.5	0.24	1.3	5.5	0.20	1.1
	167	150		6	6.5	0.21	1.4	6.0	0.17	1.0
	170	155		6	6.5	0.19	1.2	6.5	0.23	1.5
	175	170		8	7.5	0.27	2.0	8.0	0.25	2.0
	170	175		6	6.0	0.20	1.2	8.0	0.20	1.6
	205	202		6	7.0	0.19	1.3	7.0	0.21	1.5
	230	240		7	9.5	0.20	1.9	9.0	0.19	1.7
Av.	184	176		6	6.9	0.21	1.5	7.1	0.21	1.5

* Pregnant.

courages the ready acceptance of his theory that the liver of the rat is the seat of formation of ascorbic acid from carbohydrates as the ultimate source.

The experiments with the protein-fat diets also do not offer evidence in agreement with the general hypothesis. The animals on this diet thrive even worse than those on the carbohydrate diet. They consumed less food and lost considerably in weight during the experiment. The majority of the rats developed diarrhoea or soft stools soon after being placed on the diet and their intestines at the *post mortem* examination were somewhat oedematous in appearance. These organs, especially in the light group previously fasted, disclosed a rise in the wet weight (Table VI), no doubt due to the oedematous condition, and signs of loss in dry matter *etc.* reminiscent of starvation (Table IV). In fact the condition of the experimental animals was such that it would have been unsafe to assume synthesis had any increase in the concentration of the vitamin in the intestine been observed. However with the possible exception of the lighter group of the previously fasted animals, no such rise in the concentration of the ascorbic acid was observed. It should be noted that the total content in the intestines of the heavy group here was not greater than that in the lighter group, as was the case also in the similar group on the carbohydrate diet.

Nor could the rise in the intestinal concentration in rats existing on a diet of fat alone be confirmed (Table VII). These results, however, hardly deserve

Table VII.

3 days on fat diet after 2 days' fast.

Wt. of rat		Sex	Food intake g.	Wet wt. of liver g.	Ascorbic acid liver		Wet wt. of in- testine g.	Ascorbic acid intestine	
Initial g.	Final g.				mg./g.	Total content mg.		mg./g.	Total content mg.
160	142	♀	7	5.0	0.24	1.2	5.0	0.26	1.3
165	137	♀	6	4.5	0.27	1.2	7.0	0.20	1.4
165	137	♂	8	5.2	0.10	0.5	7.0	0.11	0.8
167	140	♂	6	4.5	0.27	1.2	7.0	0.20	1.4
167	142	♂	8	5.0	0.20	1.0	7.0	0.13	0.9
180	152	♂	8	5.0	0.22	1.1	7.0	0.20	1.4
Av.	167		7	4.9	0.22	1.0	6.7	0.18	1.2

serious comment. The animals in this experiment lost much more of their weight, and were in a very poor condition. At the *post mortem* examination lumps of undigested lard were found in the stomach. The livers showed definite fatty degeneration. The analysis of this organ of the rats on the diet indicated a great rise in the weight of the ether-extractable fraction (Table IV).

The results obtained in this communication do not therefore justify the hypothesis under consideration. Conceding even that on feeding a carbohydrate diet there really is a preferential rise in the indophenol-reducing capacity per unit of tissue in the liver and that a similar rise takes place in the intestine on protein or fat diets and during a fast, do such increases necessarily imply synthesis of ascorbic acid in these organs? In the first place it must be borne in mind that although, as previously shown [Zilva, 1935; Jacobsen, 1935], little doubt can exist that the capacity for reducing indophenol is in the main due to the presence of ascorbic acid in these organs, it does not necessarily follow that a rise in the reducing capacity especially of liver extracts or in extracts of oedematous tissues is due to a rise in the concentration of the vitamin. There are substances of physiological significance such as alloxantin which decolorise

Table VIII. *Average results.*

Wt. of rat		Food intake g.	Wet wt. of liver g.	Ascorbic acid liver		Wet wt. of in- testine g.	Ascorbic acid intestine	
Initial g.	Final g.			mg./g.	Total content mg.		mg./g.	Total content mg.
Stock diet (Table II).								
—	163	—	5.5	0.26	1.4	6.6	0.19	1.3
—	274	—	9.8	0.27	2.6	8.5	0.22	1.8
48 hours without food (Table III).								
177	155	—	4.7	0.26	1.2	5.6	0.26	1.4
311	281	—	7.4	0.26	1.9	7.1	0.23	1.7
3 days on carbohydrate diet after 2 days' fast (Table V).								
186	161	10	6.2	0.30	1.9	5.7	0.15	0.9
222	214	13	7.4	0.31	2.2	6.3	0.15	0.9
6 days on carbohydrate diet (Table V).								
187	163	10	5.6	0.26	1.5	5.8	0.16	0.9
3 days on protein-fat diet after 2 days' fast (Table VI).								
191	170	8	6.6	0.22	1.5	7.4	0.25	1.8
271	251	9	9.0	0.23	2.0	9.4	0.17	1.6
5 days on protein-fat diet (Table VI).								
172	151	6	6.3	0.21	1.3	6.7	0.21	1.4
12 days on protein-fat diet (Table VI).								
184	176	6	6.9	0.21	1.5	7.1	0.21	1.5
3 days on fat diet after 2 days' fast (Table VII).								
167	142	7	4.9	0.22	1.0	6.7	0.18	1.2

indophenol much more rapidly than sulphhydryl compounds in acid solution and the presence of these as well as the latter in amounts which would increase the reduction to the extent noted by Hopkins could hardly be detected by studying the velocity of the reaction as was done by his colleague G. A. Millikan. Conceding further that the increase was really due to ascorbic acid, such an increase could just as well be assumed to be due to the transfer of the vitamin from the other parts of the body to the organ in question in order to fulfil a physiological need conditioned by the constitution of the diet. Such a view is in the opinion of the writer a less hazardous speculation than that which assumes the rise in the concentration of the vitamin in an organ to be due to local synthesis induced by the consumption of a diet predominating in a certain constituent capable of acting as an ultimate precursor of the vitamin. The fact that the diets were, as seen above, so drastic in character as to bring about marked disturbances in the metabolic functions of a hardy experimental animal like the rat calls particularly for forbearance in the choice of the latter interpretation. Taking these arguments into consideration in conjunction with the observed divergencies in the essential results of the two communications, it would appear that Hopkins's hypothesis, attractive as it may be, is so far not fully supported by experimental evidence.

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CXXVI. THE OSMOTIC PRESSURE OF GLYCOGEN SOLUTIONS.

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HAWORTH and his co-workers have recently developed a chemical method for the determination of the minimum mol. wt. of polysaccharides, which involves assay of the relative amounts of tetramethyl- and trimethyl-glucoses formed by the hydrolytic cleavage of the fully methylated polysaccharide. For glycogen this method originally gave a minimum mol. wt. of about 2500 [Haworth and Percival, 1932] while that for starch was about twice this value. Later experiments however [Haworth, 1934] showed that some specimens of glycogen had mol. wts. approaching that of starch. The figure for starch is not in agreement with that determined osmotically by Samec and his collaborators, who obtained values of 100,000–500,000 [Samec and Meyer, 1921; Samec, 1934] but this and other discrepancies can be explained on the basis that the large particles in solution are aggregates of the smaller chemical molecule [Haworth, 1934].

As the development of an osmometer for low pressures [Oakley, 1935] enabled an accurate determination of the osmotic pressure of glycogen solutions to be carried out, it became of interest to compare the value for the particle weight of glycogen given by this method with the chemically determined minimum mol. wt. Furthermore as the preparation of methyl-glycogen is an essential step in Haworth's method of mol. wt. determination, and as this method has been criticised [Irvine, 1932] on the basis that the methylation process might involve some degradation of the molecule (*cf.* however Bell and Kosterlitz [1935]), a comparison of the particle weights of glycogen and of methyl-glycogen, determined osmotically, was of some interest.

Methods.

Osmometer. The special form of low pressure osmometer used in this work has been described recently [Oakley, 1935], but since further experience has indicated several improvements in design and technique, the modified form is illustrated in Fig. 1. The most important alteration is that the tap connecting the levelling tube with the main vessel is diagonally bored to eliminate the risk of a slow leak, and is placed with its axis vertical, thus enabling control to be effected by a long handle projecting from the thermostat. The levelling tube has been brought to within 1 cm. of the main vessel to reduce the angle through which the cathetometer must be turned; the alcohol manometer connection is now led off from the side of the main vessel so as to allow an unobstructed view of the menisci: the manometer is connected by a ground glass instead of a rubber joint. The tap at the top of the main vessel is now eliminated.

The temperature control must be within $\pm 0.005^\circ$.

When tap 1 is closed the liquid enters the sac *C* by osmosis and the alcohol rises to an equilibrium position in *E*. This equilibrium position fluctuates

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regularly with the temperature fluctuation of the thermostat but should not exceed 0.2 mm. Errors of parallax can be avoided by fixing a celluloid scale on to the face of the thermostat just opposite to and parallel with the capillary tube of the alcohol manometer and viewing the meniscus in line with some arbitrary mark on the scale.

The alcohol manometer reading is converted into a figure expressed as mm. of colloid solution by opening tap 1 and adjusting the level of the liquid in tube *D*, with a capillary dropper, until the alcohol meniscus attains its previous equilibrium position. It has been found that the alcohol level falls 0.1–0.2 mm. in 5 min. after adjustment and a final levelling must then be made. The difference in levels of the menisci in *C* and *D* is then read off by the cathetometer. It has been found that this “single reading” method is quicker and more reliable than the method first described [Oakley, 1935] in which an interpolation curve was plotted connecting manometer with cathetometer readings. It is advisable that the menisci in *C* and *D* should be arranged to lie in the same focal plane, since cathetometers frequently show a focussing error. Successive readings are reproducible to within 0.1 mm.

Collodion sacs were made by Adair's method [Adair, 1925].

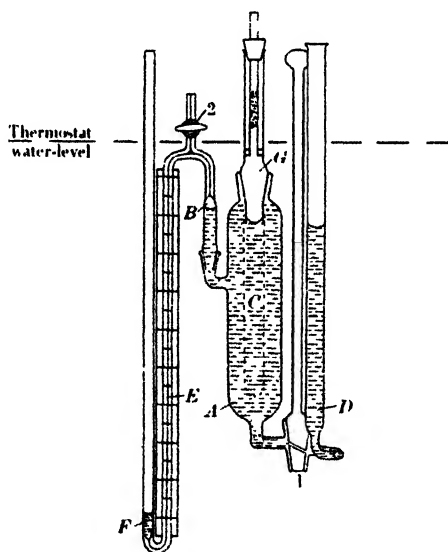


Fig. 1. *A* = cylindrical outer vessel. *G* = tube, 1 cm. in diameter, fitting into *A* by a ground glass joint. *C* = collodion sac, 10 cm. long, attached to *G*. *D* = levelling tube of the same diameter as *G*. *E* = capillary tube, 0.2 mm. in diameter, with scale attached. *F* = alcohol. *B* = meniscus and air space. Diameter of tube is 3 mm. 1 = diagonally bored tap with long handle. 2 = tap to facilitate adjustment and filling.

Preparation of glycogen. “Aqueous-extracted glycogen” and “alkali-extracted glycogen” were prepared from both liver and muscle tissue by the methods outlined by Bell and Young [1934]; in some cases further purification was effected by precipitation with 80% acetic acid as described by Bell and Young. The purity of the sample was determined in each case by the criteria laid down by these authors. All solutions were thoroughly dialysed for some days before determinations were made.

RESULTS.

Rabbit liver glycogen. Two rabbit livers from fed animals were each divided into halves and one-half of each used for the preparation of "alkali-extracted glycogen" (B), and the remainders were used for "aqueous-extracted glycogen" (A) preparation. A sample of the latter specimen was further purified by acetic acid precipitation (C).

Table I. A. *Aqueous-extracted, dried at room temperature.*

Solution	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	9.93	55.0	5.52
0.05 N CaCl ₂	9.93	28.1	2.83
0.1 N CaCl ₂	9.93	28.3	2.86
"	7.16	16.3	2.28
"	5.28	11.6	2.26
"	4.49	9.1	2.03
"	3.33	7.4	2.23
"	2.83	6.5	2.30
"	2.24	4.7	2.10
"	1.73	3.8	2.20

Average $P/C = 2.23$. $M = \text{mean mol. wt.} = 1.18 \pm 0.025 \times 10^6$.Table II. B. *Alkali-extracted, dried at room temperature.*

Solution	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	10.39	56.8	5.48
0.1 N CaCl ₂	10.39	17.4	1.68
"	8.39	11.7	1.40
"	6.02	7.15	1.19
"	4.53	5.3	1.17
"	3.42	3.95	1.16
"	2.33	2.7	1.16

Average $P/C = 1.17 \pm 0.01$. $M = \text{mean mol. wt.} = 2.16 \pm 0.02 \times 10^6$.Table III. C. *Aqueous-extracted, reprecipitated with acetic acid, dried at room temperature.*

Solution	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
0.1 N CaCl ₂	7.38	10.6	1.57
"	5.50	6.45	1.18
"	4.74	5.05	1.07
"	3.76	4.1	1.09

Average $P/C = 1.11 \pm 0.06$. $M = \text{mean mol. wt.} = 2.27 \pm 0.12 \times 10^6$.

The results are given in Tables I-III. All measurements were made at 25°. To eliminate possible effects of a Donnan membrane equilibrium resulting from the presence of any ionogenic groups in the colloid, experiments were carried out using $N/10$ CaCl₂ as solvent. That such a precaution is necessary is shown by the large drop in osmotic pressure when CaCl₂ solution is substituted for pure

water. Such a fall in osmotic pressure might be due to increased aggregation of the particles; if this were so, however, the average degree of aggregation would not be expected to remain independent of concentration but to decrease with decreasing concentration, thus giving increasing rather than constant values for P/C .

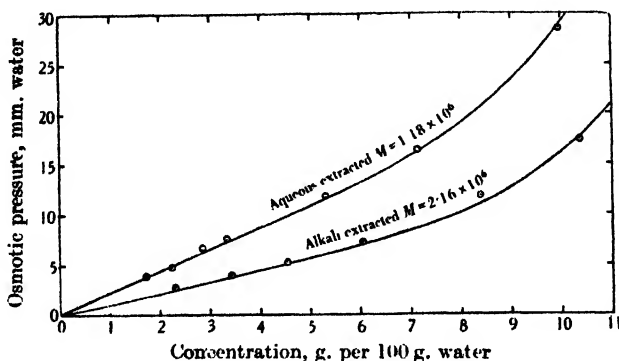


Fig. 2. Osmotic pressure of solutions of liver glycogen in 0.1 N CaCl_2 .

The apparent "mean mol. wt.", or average particle weight, was calculated from the equation used by Adair:

$$M = 10RT \cdot C/P = 2,520,000 \cdot C/P \text{ at } 25^\circ$$

where M = mol. wt., C = concentration of glycogen in 100 g. water, P = osmotic pressure in mm. water at 4° . The concentration was estimated by weighing the residue after evaporating the solution *in vacuo* at 100° , and subtracting the weight of CaCl_2 calculated to be present on the assumption that it was present in the same concentration relative to water on both sides of the membrane.

The data of Tables I and II are plotted in Fig. 2. A good straight line is obtained below a concentration of 6%.

Samples B and C both have "mean mol. wt." of about 2,000,000, while sample A has a value of about 1,000,000. It seems probable that this lower value is due to the presence of a small quantity of an impurity of much lower mol. wt., since precipitation with acetic acid considerably raised the value as shown by sample C.

Alkali-extracted rabbit muscle glycogen. The preparation was dialysed for 2 days and the osmotic pressure then measured against water and $N/50$ NaCl with the results given in Table IV and Fig. 3.

Table IV. *Rabbit muscle glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	3.18	121.4	38
$N/50$ NaCl	3.18	20.3	6.4
	2.47	12.7	5.3
	1.51	6.4	4.2
	1.00	4.0	4.0
	0.64	2.5	3.9
	0.45	1.65	3.7

$$P/C \text{ (limit)} = 3.6.$$

$$M = \text{mean mol. wt.} = 0.7 \times 10^6.$$

The results were much less satisfactory than those with liver glycogen and it appeared that the concentration of NaCl was too low completely to repress ionic pressure effects. For this reason the experiment was repeated with $N/10$ CaCl_2 as solvent with the results given in Table V and Fig. 3.

Table V. *Rabbit muscle glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
$N/10$ CaCl_2	4.25	12.3	2.9
"	2.29	5.3	2.3
"	1.47	3.3	2.2
"	1.13	2.7	2.4
"	0.72	1.0	2.6

P/C (limit) = 2.3.

M = mean mol. wt. = 1.1×10^6 .

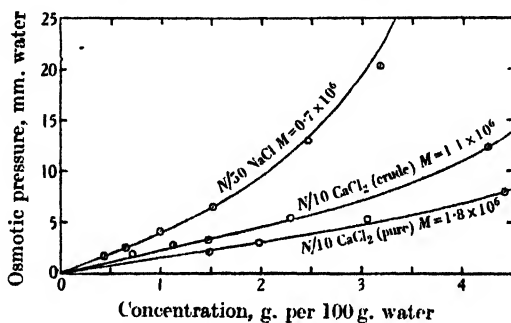


Fig. 3. Osmotic pressures of muscle glycogen solutions.

The P/C curve is lower and flatter than in the previous experiment, showing that the effect of ionic pressure has been almost suppressed, although the last two points are high.

Acetic acid-precipitated muscle glycogen. An alkali-extracted specimen of rabbit muscle glycogen was precipitated with acetic acid and again treated with KOH followed by another acetic acid precipitation and dialysis. The results are given in Table VI and Fig. 3.

Table VI. *Rabbit muscle glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
$N/10$ CaCl_2	4.43	7.9	1.78
"	3.06	5.3	1.73
"	1.91	2.9	1.55
"	1.48	2.1	1.42

P/C = 1.40.

M = mean mol. wt. = 1.8×10^6 .

The curve for P/C is still lower and flatter than in the previous experiment giving a mean particle weight of nearly 2×10^6 and it seems probable that there is no significant difference between the apparent mol. wt. of the specimens of liver and muscle glycogen used, but that the latter is much more difficult to free from impurities and is admixed with substances of lower mol. wt. which can be removed only with difficulty.

Methyl-glycogen in water. We are greatly indebted to Dr D. J. Bell of the Department of Physiology, University of Aberdeen, for a sample of methyl-glycogen prepared according to the method of Haworth and Percival [1932] from rabbit liver glycogen [Bell, 1935]. This specimen had a methoxyl content of 45.1% and was readily soluble in cold water. The osmotic data are given in Table VII and Fig. 4.

Table VII. *Methyl-glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	9.37	172	18.4
N/10 CaCl ₂	9.37	51.1	5.47
"	6.44	24.7	3.83
"	4.23	12.0	2.83
"	2.7	6.0	2.22
"	1.76	3.5	1.99
"	1.14	2.15	1.89

P/C (limit) = 1.85.

M = mean mol. wt. = 1.36×10^6 .

As appears from Fig. 4, P/C does not reach its limiting value until a concentration of about 1% is reached. The mean particle weight is of the same order as that of glycogen itself, the value after correction for methyl content being 1.08×10^6 .

Methyl-glycogen in benzene. It might be expected that a change from water to an organic solvent would profoundly alter any micellar equilibrium should this exist, and the possibility that benzene might favour the dispersion of the highly methylated compound, owing to the affinity between the aliphatic methyl groups and the organic solvent, arose.

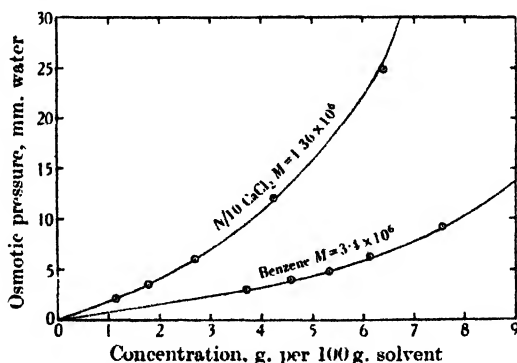


Fig. 4. Osmotic pressure of solutions of methyl-glycogen.

Collodion membranes were used, being transferred first to water, then to alcohol and finally to benzene by way of alcohol-benzene mixtures. As stopcock lubricant for the osmometer metaphosphoric acid was used, this being protected from the water of the thermostat by rings of ordinary stopcock grease in appropriate positions. Owing to the large coefficient of expansion of benzene the fluctuation of the alcohol meniscus in the manometer amounted to about 1 mm. so that it was hardly possible to take readings below 3 mm.; in spite of this the results lie on a good curve as shown in Fig. 4 and Table VIII. The particle weight corrected for methyl content is 2.7×10^6 and although it is about $2\frac{1}{2}$ times

that in water it is still of the same order of magnitude. Carter and Record [1936] have recently found that the osmotic pressure of methyl-glycogen in carbon tetrachloride or nitromethane indicates an average particle weight of the order of 1×10^6 , a value not greatly different from those which we have observed in aqueous or benzene solution.

Table VIII. *Methyl-glycogen in benzene.*

C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
7.57	9.15	1.21
6.10	6.25	1.03
5.33	4.75	0.89
4.57	3.97	0.85
3.70	3.00	0.81

P/C (limit) = 0.75.

M = mean mol. wt. = 3.4×10^6 .

Apparent electrovalency. If we assume that the value of P/C associated with the osmotic pressure of the colloidal particles only is equal to the observed limiting value of P/C in CaCl_2 solution, i.e. the value of P/C when $C=0$, and is constant for all concentrations of glycogen, then the observed rise of the value of P/C in CaCl_2 solution with increasing concentration might be due to the influence of ions, the effect of which becomes appreciable only at colloid-ionic concentrations which are significant in comparison with the concentration of Ca^{++} and Cl^- ions.

The number of ions dissociated per glycogen particle at concentration c would then be given by

$$\frac{\left(\frac{P}{C}\right)_c \text{ water} - \left(\frac{P}{C}\right)_0 \text{ (CaCl}_2\text{)}}{\left(\frac{P}{C}\right)_0 \text{ CaCl}_2},$$

where $\left(\frac{P}{C}\right)_c \text{ water}$ = the value of $\frac{P}{C}$ in water at concentration c , and

$\left(\frac{P}{C}\right)_0 \text{ CaCl}_2$ = the value of $\frac{P}{C}$ in CaCl_2 at concentration equal to zero.

In Table IX are given the values thus calculated for the apparent electrovalency of glycogen, i.e. the number of ions dissociating from each particle of glycogen.

Table IX.

Substance	Concentration %	Apparent electro- valency
Liver glycogen A	9.93	1.5
Liver glycogen B	10.39	3.7
Muscle glycogen	3.18	16.0
Methyl-glycogen	9.37	9.0

It is impossible to say whether this charge is associated with the real structure of the molecule, for example the dissociation of a phosphoric acid group, or is due to an adsorbed or free impurity; it may merely illustrate the fact that even chemically neutral particles may show a charge in water, e.g. air or amyl alcohol. It must be remembered that a charge of a few units on a particle weighing some 2,000,000 represents an exceedingly small stoichiometrical quantity.

DISCUSSION.

The data obtained show that treatment with potassium hydroxide and precipitation with 80 % acetic acid have little, if any, significant effect on the particle weight of rabbit liver glycogen in aqueous solution. The value of about 2×10^6 obtained here is significantly different from that obtained by Samec and Isajevic [1923], who found a value for the micellar magnitude of dog liver glycogen in water of about 114,000.

The value of about 2×10^6 for the mean particle weight of rabbit liver glycogen in aqueous solution is in agreement with that given in Table VI for highly purified rabbit muscle glycogen although a difference of the particle size might have been suspected on the grounds that the specimen of muscle glycogen used gave, as do many muscle glycogen preparations, a distinct purplish tint with iodine, quite different from the brown colour given by liver glycogen. This common, though not universal property of muscle glycogen preparations may depend on the length of the chain of the chemical molecule rather than on the particle size in solution. The difference in colour with iodine was the main distinction in properties between the specimens of muscle glycogen used in this investigation and liver glycogen, as determined by the criteria laid down by Bell and Young [1934].

Methylation of glycogen seems to have little effect on the size of the particles in aqueous solution, although one might expect that if the glycogen particle in water consists of an aggregate of large numbers of chemical molecules, the substitution of hydrophilic hydroxyl groups by hydrophobic methoxyl groups might have a marked effect on the state of aggregation in aqueous solution. Furthermore, the fact that the particle size of methyl-glycogen in benzene is of the same order of magnitude as that in water seems to indicate that the aggregating forces may be different from those concerned with known cases of physical aggregation, which are usually modified by a change from aqueous to organic solvents.

If fully methylated glycogen gives an aggregated particle of the same order of magnitude as that of non-methylated glycogen then those hydroxyl groups which are accessible to methylation cannot be responsible for the aggregation [Haworth, 1929]. Bell [1935], however, has recently shown that methyl-glycogen, with a methoxyl content of 45.5 %, contains two free hydroxyl groups in each dodecasaccharide unit so that it seems possible that these unmethylated hydroxyl groups are concerned with the aggregating forces. Bell has, however, introduced nitro- and dichloroacetyl groups into the free hydroxyl groups of apparently fully methylated glycogen and found that the properties of the compound were not affected in any obvious manner, but the criteria which he used were not such as would easily indicate a change of aggregation. If these fully substituted compounds were shown to possess a much higher degree of dispersion, this would be strong evidence that the two non-methylated hydroxy-groups in ordinary methyl-glycogen are responsible for the aggregation, and a plausible hypothesis would be that they induce aggregation by engaging in co-ordinated oxygen bridges both in methylated and in ordinary glycogen. This would explain why the degree of aggregation of methyl-glycogen is not significantly affected by a change of solvent. Osmotic pressure determinations with solutions of methyl-glycogen in mixed solvents should be made, however, since greater dispersion is sometimes obtained in such solutions than with solutions in pure solvents.

The values for the "molecular weight" given in this paper are, of course, only mean values and we have no evidence whether glycogen in solution is

mono-disperse or not. Lamm [1934], using the ultracentrifuge, showed that certain preparations of starch were polydisperse; whether glycogen exhibits a similar behaviour can be shown only by a similar means.

SUMMARY.

1. Solutions of rabbit liver glycogen, rabbit muscle and methylated rabbit liver glycogen in $N/10$ CaCl_2 all give osmotic pressures indicating a mean particle weight of the order of 2×10^6 .

2. A solution of methyl-glycogen in benzene gave an osmotic pressure indicating a larger particle size but of the same order of magnitude (3.4×10^6).

3. The significance of these results is discussed.

We are greatly indebted to Dr D. J. Bell of the University of Aberdeen for a gift of methyl-glycogen. The cost of preparing the glycogen used in this research was defrayed from a grant to one of us (F. G. Y.) from the Government Grants Committee of the Royal Society, to which our thanks are due. One of us (H. B. O.) also wishes to thank Prof. F. G. Donnan for his kindly interest and Messrs Unilever Ltd. for a grant during a part of this work.

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CXXVII. FUMARATE AND TISSUE RESPIRATION.

I. EFFECT OF DICARBOXYLIC ACIDS ON THE OXYGEN CONSUMPTION.

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MALONATE strongly inhibits the enzymic dehydrogenation of succinate [Quastel and Wooldridge, 1928; Quastel and Wheatley, 1931]. Gözsy and Szent-Györgyi [1934] found that it also strongly inhibits the respiration of minced pigeon breast muscle. They considered that in this tissue a water-soluble substance acted as hydrogen carrier between activated substrate and activated oxygen, and that this substance was succinate or fumarate. As a result of further work on the same tissue by Szent-Györgyi and his colleagues this idea was elaborated [Annau *et al.*, 1935]. The hydrogen carrier is fumarate, which, activated by a dehydrogenase, is oxidised to oxaloacetate by the "Warburg-Keilin system", *i.e.* by cytochrome respiratory enzyme and oxygen. The oxaloacetate is immediately reduced back to fumarate (or malate) by the activated substrates. The inhibition of respiration by malonate was explained as follows.

During respiration a small fraction of the oxaloacetate formed is "over-reduced" to succinate. This would represent a loss of the fumarate which is acting as a hydrogen carrier were not the succinate promptly oxidised to fumarate by succinoxidase. In the presence of malonate the succinoxidase is inhibited, so that succinate accumulates, fumarate disappears, hydrogen transport fails, and the respiration falls.

The experiments described in this paper represent part of a critical study of Szent-Györgyi's theory which will attempt:

- (1) to confirm the experimental basis of the theory;
- (2) to find out whether fumarate can act as hydrogen carrier in tissues other than pigeon breast muscle; and
- (3) to determine the nature of the activated substrates which can transfer their hydrogen to oxaloacetate, *i.e.* to see whether the theory applies to the oxidation of fat, protein or carbohydrate.

This paper deals with the effects of dicarboxylic acids on the respiration. In considering whether these effects accord with Szent-Györgyi's views the following must be borne in mind.

Firstly, it is not essential that added fumarate should accelerate the respiration of the tissue. This should only occur when the tissue contains less than the optimum amount of fumarate, either normally or as a result of loss by diffusion into the suspension fluid. Secondly, it is not essential that malonate should inhibit the respiration; this should only take place when the reduction intensity of the tissue is high enough for "over-reduction" of oxaloacetate to succinate to occur. Thirdly, if malonate does inhibit the respiration, this inhibition should be prevented by the simultaneous addition of fumarate.

¹ Halley Stewart Research Fellow.

EXPERIMENTAL METHODS AND RESULTS.

Measurements of oxygen consumption were made in the Haldane-Barcroft-Warburg apparatus. The suspension medium was buffered by sodium phosphates, p_H 7.3.

Preparation of tissue. Immediately after the death of the pigeon, the breast muscle was cut into several pieces, which were quickly cooled on distilled-water ice. After quick drying on filter-paper, the pieces were passed through an ice-cold mincer into an ice-cooled dish. The mincer forced the tissue by a plunger through a sieve plate and cut it on the far side by revolving knives, as specified by Annau *et al.* [1935]. In some experiments the minced muscle was weighed and added to 3 vols. ice-cold phosphate buffer; the suspension was then pipetted into the Warburg vessels. In other experiments, equal portions of tissue were quickly weighed on a torsion balance and transferred to the Warburg vessels. The vessels described by Dickens and Šimer [1930, 1] were found to be suitable, provided that the oxygen uptake did not exceed 500 μ l. per hour. 100 mg. tissue (moist weight) were used in all experiments with pigeon breast muscle. The vessels were filled with air.

In experiments with tissue slices, the tissue was prepared in solutions on ice or at 38°, according to the circumstances; the vessels were filled with oxygen, sometimes while inside the thermostat, sometimes while outside.

Effects of malonate and fumarate on the respiration of minced pigeon breast muscle.

Banga [Annau *et al.*, 1935] found that with fumarate addition the muscle showed a large constant oxygen uptake; without addition, the respiration was either almost the same as that with fumarate, or, more usually, it progressively decreased. She concluded that "das Fumarat die Atmung eigentlich nicht 'steigert', sondern bloss 'konserviert'; vom Abfall bewahrt". Malonate caused a strong inhibition; with fumarate and malonate the respiration was the same as with fumarate alone. These observations have been confirmed.

The respiration of the tissue without fumarate or malonate addition depends on the osmotic pressure of the suspension fluid (Figs. 1 and 2). In the experiment of Fig. 1 the muscle was placed in a hypotonic solution. The solutions in the vessels were as follows:

	<i>F</i> ml.	<i>S</i> ml.	<i>N</i> ml.
Phosphate (0.11 <i>M</i>)	0.5	0.5	0.5
Fumarate (0.1 <i>M</i>)	0.4	—	—
NaCl (0.9 %)	—	0.4	—
Water	1.1	1.1	1.5

The solutions corresponding to the curves of Fig. 2 were very slightly hypertonic, and were as follows:

	<i>F</i> ml.	<i>N</i> ml.	<i>M</i> ml.
Phosphate (0.177 <i>M</i>)	0.3	0.3	0.3
Fumarate (0.1 <i>M</i>)	0.4	—	—
Malonate (0.1 <i>M</i>)	—	1.6	0.2
NaCl (0.9 %)	1.2	1.6	1.4

In the second experiment the respiration did not fall off in the absence of added fumarate. Malonate (0.01 *M*) inhibited strongly. In the first experiment (hypotonic solutions) the respiration was low and falling. It was maintained at a high value when 0.02 *M* fumarate was added. When instead of fumarate NaCl was added in iso-osmotic concentration (0.03 *M*) the respiration was again

maintained at a higher value, even if not quite so well as by the fumarate. Part of the effect of the fumarate might, therefore, be ascribed to osmotic action. This consideration is not likely to affect the interpretation of Banga's experiments which were performed in solutions which were not very hypotonic

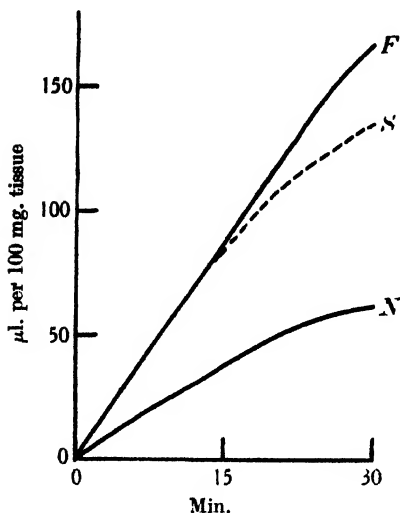


Fig. 1. Pigeon breast muscle (hypotonic solution).

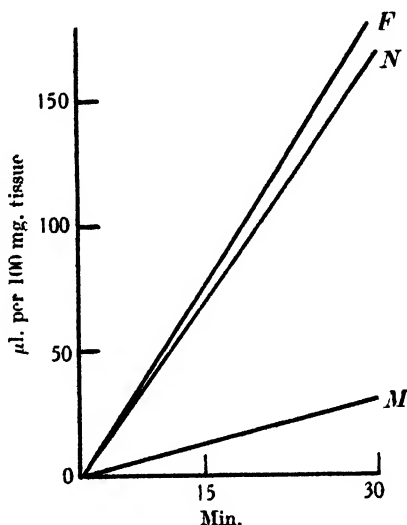


Fig. 2. Pigeon breast muscle (slightly hypertonic solution).

N, no addition; F, fumarate; M, malonate; S, NaCl.

(0.05 *M* phosphate). But in the experiment of Gözsy and Szent-Györgyi [1934], in which it was shown that fumarate raised the respiration without itself disappearing, the muscle was suspended in 0.022 *M* phosphate, and in view of this, the experiment loses much of its force.

The effect of calcium.

When the respiration of minced pigeon breast muscle is measured in isotonic phosphate-containing solutions, the addition of calcium in the concentration in which it occurs in blood or Ringer's solution causes a strong inhibition of the respiration. In the experiment shown in Fig. 3, the effect of the addition of 8.8 mg./100 ml. Ca and 20 mg./100 ml. K is shown. The solutions (1.9 ml. in each vessel) were isotonic, the phosphate concentration was *M*/36, and the curves correspond to the following additions:

	Fumarate (<i>M</i>)	Malonate (<i>M</i>)	Calcium (mg./100 ml.)	Potassium (mg./100 ml.)
<i>F</i>	0.02	—	—	—
<i>M</i>	—	0.01	—	—
<i>CF</i>	0.02	—	8.8	20
<i>CFM</i>	0.02	0.01	8.8	20
<i>CN</i>	—	—	8.8	20
<i>CM</i>	—	0.01	8.8	20

On the addition of calcium and potassium, fumarate and malonate have little effect on the respiration, which becomes very low and of the same order as the

malonate-inhibited respiration in the absence of calcium. A number of experiments showed that the effect was due to the calcium alone, the respiration being strongly inhibited by concentrations as low as 4 mg. per 100 ml.

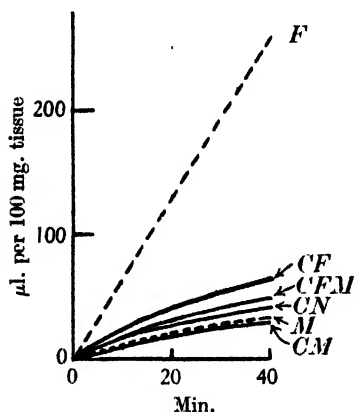


Fig. 3.

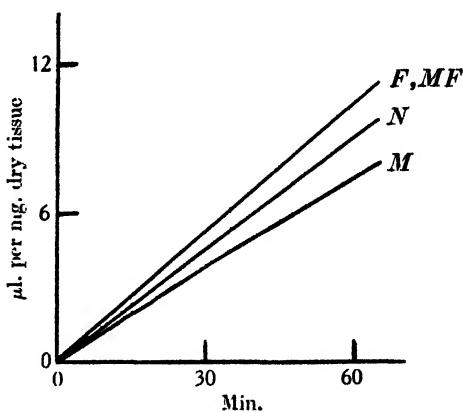


Fig. 4.

Fig. 3. Pigeon breast muscle.

Fig. 4. Rat diaphragm. 0.002 *M* CaCl_2 , 0.005 *M* KCl . *N*, no addition; *F*, 0.02 *M* fumarate; *M*, 0.01 *M* malonate; *MF*, 0.01 *M* malonate + 0.02 *M* fumarate.

That calcium strongly inhibits the respiration of minced tissue was discovered by Thunberg [1909, 1, 2] and his observations have been confirmed and extended by others, including Meyerhof [1919] and Holck [1934]. It is now seen that this inhibition also occurs in the presence of fumarate.

It is interesting to compare the effects of cations on the minced pigeon muscle with their effects on the respiration of slices of rat brain cortex [Dickens and Greville, 1935]. With the latter material, calcium lowers the respiration, potassium accelerates it [compare Ashford and Dixon, 1935] and can overcome the effect of calcium. Magnesium also lowers the respiration. Experiments with minced pigeon breast muscle in isotonic solutions containing 0.02 *M* fumarate have shown that: (1) Potassium (concentrations up to 0.09 *M*) has little effect on the respiration. (2) Addition of potassium (0.003–0.15 *M*) does not relieve the inhibition due to calcium. (3) Magnesium (up to 0.005 *M*) has little effect on the respiration.

Dickens and Greville [1935] have suggested that calcium modifies the condition of the brain protoplasm so as to make the enzymes less available. If, as seems probable, the effect of calcium on the minced muscle is due to irreversible damage consequent on penetration of the calcium into the tissue, the differences in the effects of cations in the two materials are not surprising.

It is clear that the effects of the dicarboxylic acids have been studied using tissue in such an "unphysiological" condition that the respiration is heavily inhibited by the addition of "physiological" concentrations of calcium.¹

¹ It may be thought that the sensitivity of the tissue towards calcium in these experiments is due to damage caused by the particular mincer used. The respiration in Szent-Györgyi's experiments in calcium- (and bicarbonate-) containing Ringer solution is only a little lower than that in the calcium-free phosphate. There is, however, no evidence of undue damage. The mincer conformed to Szent-Györgyi's specifications, and the oxygen uptakes observed in absence of added fumarate agree well with those given by Straub [Annan *et al.*, 1935].

Ahlgren [1925] remarks that "Ringer-Lösung für feinverteilte Gewebe wenigstens bei Atmungsversuchen eine sehr ungeeignete Suspensionsflüssigkeit ist". The more acceptable view would be that it is the state of the tissue rather than the fluid which is unsuitable. Hence it seemed desirable to test the effects of malonate and fumarate on tissue as little damaged as possible.

Effects of dicarboxylic acids on the respiration of diaphragm and heart muscle.

The most suitable material seemed to be the rat diaphragm. Warburg *et al.* [1924] showed that with young rats (100 g. or less) the tissue is thin enough to be completely saturated with oxygen; and Meyerhof and Himwich [1924] and Meyerhof *et al.* [1925] found that respiration is constant for 2 or 3 hours, being somewhat increased by the addition of pyruvate.

The diaphragms of rats of 80–100 g. were freed from the central tendon and divided into four. The respirations of the four pieces agreed well. Fig. 4 shows that in isotonic media containing phosphate, calcium and potassium, 0.01 *M* malonate has little effect on the respiration. In the absence of Ca and K similar curves were obtained: calcium did not inhibit the respiration. Malonate had no greater effect in the presence of pyruvate. But if, in the absence of added substrate, higher concentrations of malonate be added, and the respiration followed over longer periods, malonate is seen to exert a very definite effect (Fig. 5). Fig. 6 shows that 0.02 *M* fumarate overcomes the inhibiting action of

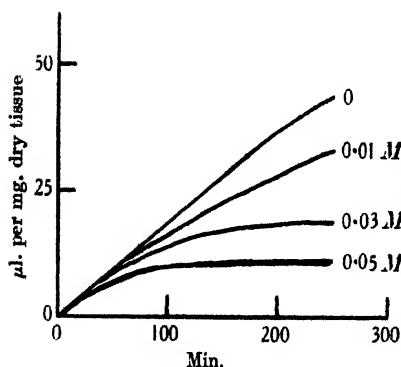


Fig. 5.

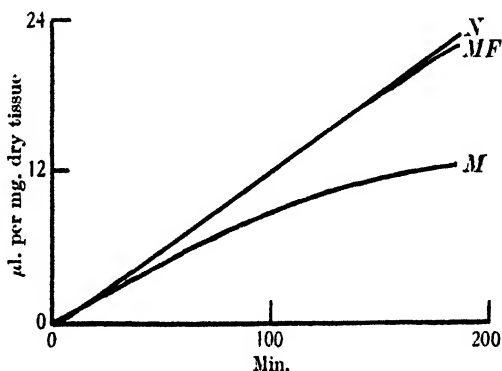


Fig. 6.

Fig. 5. Rat diaphragm. Inhibition by malonate.

Fig. 6. Rat diaphragm. *N*, no addition; *M*, 0.03 *M* malonate; *MF*, 0.03 *M* malonate + 0.02 *M* fumarate.

0.03 *M* malonate. Thus the events which are explained by Szent-Györgyi's theory can occur with tissue that has suffered minimum damage, the respiration of which is not reduced by the usual concentrations of calcium.

If now the diaphragm tissue be severely damaged, by being cut into small pieces, the lower concentration of malonate (0.01 *M*) has an immediate action, the respiration falls off in the absence of added fumarate, and calcium strongly inhibits the respiration in the presence of fumarate. Fig. 7 shows an experiment in which the pieces of diaphragm were weighed on a torsion balance, and then each piece rapidly cut with scissors to a fine brei. Control experiments showed that the respirations of four pieces of the same diaphragm cut in this way agreed

well. If the damage were not so severe, the cut particles being larger, the respiration did not fall off so rapidly in the absence of fumarate, calcium had not much effect, but malonate still inhibited strongly (Fig. 8).

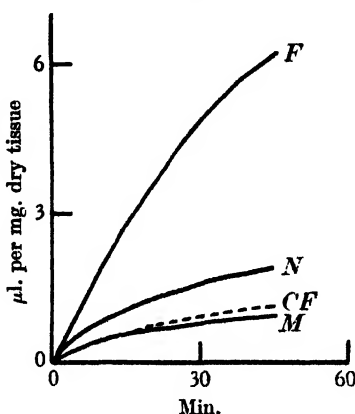


Fig. 7.

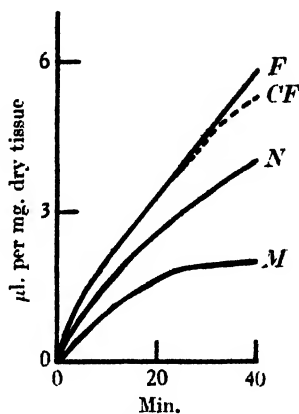


Fig. 8.

Fig. 7. Rat diaphragm. Severely damaged. Isotonic solutions. *F*, 0.01 *M* fumarate; *M*, 0.01 *M* malonate; *N*, no addition; *CF*, 0.01 *M* fumarate + 0.002 *M* CaCl_2 + 0.005 *M* KCl .

Fig. 8. Rat diaphragm, moderately damaged. (Legend as in Fig. 7.)

Finally, with muscle tissue cut in slices the effects of malonate and fumarate were very pronounced. This is shown by an experiment with sliced guinea-pig heart muscle:

	1	2	3	4
Phosphate (0.11 <i>M</i>)	0.6	0.6	0.6	0.6
Fumarate (0.1 <i>M</i>)	—	—	0.4	0.4
Malonate (0.1 <i>M</i>)	—	0.2	—	0.2
NaCl (0.9 %)	1.4	1.2	1.0	0.8
QO_2 (first 40 min.)	-5.5	-1.5	-13.7	-14.8

Effect of dicarboxylic acids on the respiration of brain cortex and retina.

As a first step towards finding out whether carbohydrates can be oxidised in the tissues by means of fumarate catalysis, the effects of fumarate and malonate on tissues with a pure carbohydrate respiration have been tested. One such tissue is the brain cortex, the respiration of which is maintained only when it is supplied with¹ carbohydrates or carbohydrate derivatives, which are burnt completely [Loebel, 1925; Dickens and Simer, 1930, 2]. Fig. 9 shows the effect of 0.05 *M* fumarate and 0.02 *M* malonate on the respiration of rat brain cortex slices in glucose and phosphate-containing medium. Fumarate accelerates the respiration; malonate reduces it to below half the control value. It may remain constant at this lowered value for at least 90 min. Fumarate only partially relieves the malonate inhibition. Similar results are obtained whether the tissue is prepared in solutions at room temperature, at 38°, or on ice.

Malonate has less effect on the respiration when the substrate is pyruvate than when it is glucose. Fig. 10 shows the effect of 0.01 *M* malonate on the oxygen consumption of rat brain cortex in the presence of 0.2 % glucose, of 0.2 % lithium

¹ Succinic, α -ketoglutaric and *l*(+)-glutamic acids are also vigorously oxidised [Quastel and Wheatley, 1932; Krebs, 1935; Weil-Malherbe, 1935].

pyruvate and of 0.2 % glucose + 0.2 % lithium pyruvate. If Szent-Györgyi's explanation of inhibition by malonate is to apply to brain cortex, it would be necessary to say that since malonate has less effect on the respiration, in the

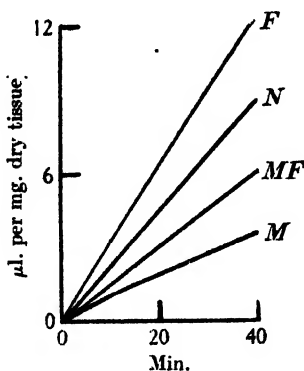


Fig. 9.

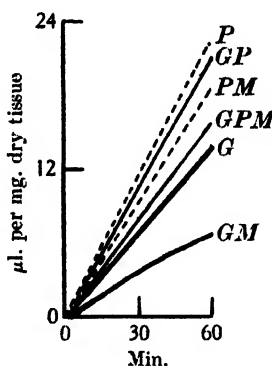


Fig. 10.

Fig. 9. Rat brain cortex (slices). *N*, no addition; *M*, malonate; *F*, fumarate; *MF*, malonate + fumarate.

Fig. 10. Rat brain cortex (slices). *G*, glucose; *GM*, glucose + malonate; *P*, pyruvate; *PM*, pyruvate + malonate; *GP*, glucose + pyruvate; *GPM*, glucose + pyruvate + malonate.

presence of pyruvate than in the presence of glucose, the reducing intensity (or the "Wasserstoffmobilisierung") is less when the former is substrate [Annau *et al.*, 1935, p. 12]. This would be possible. Again, since addition of pyruvate lessens the malonate inhibition in the presence of glucose (Fig. 10), addition of pyruvate to the glucose solution would have to lessen the "Wasserstoffmobilisierung" and the "Wasserstoffüberschuss" of the tissue; and a consideration of molecular collisions shows this to be possible also.

It will be seen from Fig. 9 that the addition of 0.02 *M* fumarate reduces the inhibition due to 0.01 *M* malonate, but that the relief is partial. It is essential, if Szent-Györgyi's explanation is to apply, that fumarate should relieve the malonate inhibition completely; but it is possible that a failure of the fumarate to diffuse adequately into the tissue prevents this from being observed. Evidence has been sought in several ways:

1. *Attempted alterations in the permeability of tissue.* The respiration of brain cortex is very dependent on the cations in the medium [Dickens and Greville, 1935]. The medium in the experiment of Fig. 9 contained Na, Ca, K, Mg. Removal of Ca, of Ca and Mg or of Ca, K and Mg did not appreciably modify the effects of fumarate and malonate (Table I; in these experiments the

Table I. — Q_{O_2} of rat brain cortex.

Glucose-phosphate-medium. Cation concentrations as in the paper by Dickens and Greville [1935].

Cations	Duration min.	No addition	Malonate (0.01 <i>M</i>)	Fumarate (0.02 <i>M</i>)	Malonate (0.01 <i>M</i>) + Fumarate (0.02 <i>M</i>)
Na-K-Ca-Mg	40	14.0	6.3	15.5	10.4
Na-K-Mg	30	14.2	5.8	17.2	9.8
Na-K	35	19.4	7.7	21.6	12.2
Na	40	19.1	7.5	20.8	10.5

fumarate and malonate concentrations were the same as those in the pigeon breast muscle experiment).

2. *Cut tissue.* With diaphragm, cutting the tissue resulted in more marked actions by malonate and fumarate. When rat brain cortex slices were cut with scissors to a brei, the effect of malonate became rather less; and no evidence was obtained of increased relief by fumarate.

3. *Increased fumarate concentrations.* Relief was still only partial with 0.06 *M* fumarate (Table II).

4. *Malate.* Instead of fumarate, the effect of *l*-malate was tried. This should be brought into equilibrium with fumarate by the tissue fumarase [Quastel, 1931], and might possibly penetrate better. However, the relief was still incomplete (Table II).

Table II.

Substrate: glucose. Time: 60 min.					
Malonate (<i>M</i>)	—	0.01	0.01	0.01	0.01
Fumarate (<i>M</i>)	—	—	0.06	—	—
<i>l</i> -Malate (<i>M</i>)	—	—	—	0.01	0.03
— Q_{O_2}	17.5	5.4	12.6	9.2	13.0
Malonate (<i>M</i>)	—	0.01	0.01	0.01	—
<i>l</i> -Malate (<i>M</i>)	—	—	0.02	0.04	—
— Q_{O_2}	17.7	7.3	13.4	14.4	—

In order to see whether this imperfect relief was a peculiarity of brain tissue, the behaviour of another tissue with pure carbohydrate respiration was tested. With rat retina, fumarate was even less effective:

Substrate: glucose.			
	No addition	Malonate (0.01 <i>M</i>)	Malonate (0.01 <i>M</i>) + fumarate (0.02 <i>M</i>)
— Q_{O_2} (60 min.)	21.4	6.1	7.4

Tumour tissue.

It was found that 0.02 *M* malonate inhibited the respiration of slices of Jensen rat sarcoma in glucose medium by 20–40 %. Similar results have recently been published by Boyland and Boyland [1936].

Effect of malonate and fumarate on artificially-stimulated respiration.

Dinitrophenols accelerate the respiration of normal tissues [Dodds and Greville, 1933; Ehrenfest and Ronzoni, 1933; Euler, 1933] and tumour tissues [Dodds and Greville, 1934]. The increased respiration is strongly inhibited by malonate. In the following experiment with rat brain cortex, malonate and fumarate were added after 30 min. respiration (substrate: glucose).

Dinitro- <i>o</i> -cresol present initially ($M \times 10^{-5}$)	—	3	3	3	3
Added after 30 min.	—	—	Malonate (0.02 <i>M</i>)	Fumarate (0.02 <i>M</i>)	Malonate (0.02 <i>M</i>) + fumarate (0.02 <i>M</i>)
— Q_{O_2} before addition (30 min.)	13.1	39.0	36.3	36.6	33.2
— Q_{O_2} after addition (40 min.)	13.7	40.6	11.9	38.5	13.9

The next experiment, in which dinitro-*o*-cresol and malonate were added together, also shows that the latter abolishes the action of the former on the

respiration of rat brain cortex. The respiration during 30 min. after addition is expressed as a percentage of that during 30 min. before addition.

Fumarate present initially (<i>M</i>)	—	—	—	0.02	0.08
Added after 30 min.:					
Dinitro- <i>o</i> -cresol (<i>M</i> × 10 ⁻⁵)	—	3	3	3	3
Malonate (<i>M</i>)	—	—	0.01	0.01	0.01
% of initial respiration	101	184	62	78	98

Concentrations of fumarate as high as 0.08 *M* do not overcome the action of malonate in preventing the acceleration.

In tumour tissue (JRS) malonate prevents the acceleration by dinitro-*o*-cresol in the same way. In the experiment given below, malonate and dinitro-*o*-cresol were added after a preliminary period. The respiration after addition (35 min.) is expressed as a percentage of the respiration before addition (35 min.). With both brain and JRS, dinitro-*o*-cresol only accelerates the respiration in presence of carbohydrate or carbohydrate derivatives; so presumably here we have another case of carbohydrate respiration being inhibited by malonate.

Besides the nitrophenols, certain of the redox indicators accelerate the respiration of tumour tissue [Barron, 1930; Dickens, 1934]. The acceleration in respiration produced by one of the most efficient of these, brilliant cresyl blue [Dickens, unpublished], is not prevented by malonate.

JRS. Substrate: glucose.

Added after 35 min.:					
Malonate (<i>M</i>)	—	—	0.01	—	0.01
Dinitro- <i>o</i> -cresol (<i>M</i> × 10 ⁻⁶)	—	1	1	—	—
Brilliant cresyl blue (<i>M</i> × 10 ⁻⁴)	—	—	—	2	2
% of initial respiration	89	204	85	218	207

Although their effects are often similar, the modes of action of the redox indicators and nitrophenols on respiration are possibly quite different [Greville and Stern, 1935; see also Krahl and Clowes, 1935]. That malonate overcomes the action of the latter and not of the former may therefore be of interest.

DISCUSSION.

According to Szent-Györgyi, fumarate acts as a hydrogen carrier for the tissue oxidations. In any particular tissue it might act as a hydrogen carrier for the whole of the respiration or for a part of the respiration. Before the former could be claimed for a given tissue it would be necessary to establish that:

(1) The tissue, killed at any time during full respiration in such a way that *post mortem* chemical changes could not occur, contains enough dicarboxylic acid (fumaric + malic + succinic + oxaloacetic) to be a catalyst for the whole respiration at that time.

(2) The tissue's own fumarate (or malate) can be oxidised to oxaloacetate in the tissue, and the tissue's own oxaloacetate can be reduced to fumarate (or malate) in the tissue.

(3) The tissue's own fumarate (or malate) is oxidised to oxaloacetate at a rate at least equivalent to the oxygen uptake during respiration.

(4) The tissue's own oxaloacetate is reduced to fumarate (or malate) at a rate at least equivalent to the oxygen uptake.

(5) Oxaloacetate reduction in the tissue is connected with substrate oxidation.

These conditions are essential, but it would be almost impossible to establish (3), (4) and (5). If the following further conditions, which are in themselves not essential, could be established in addition to (1) and (2), it would become highly probable that fumarate acts as a catalyst for the whole of the respiration.

(6) Added fumarate (or malate) is oxidised to oxaloacetate at a rate at least equivalent to the oxygen uptake during respiration.

(7) Added oxaloacetate is reduced to fumarate (or malate) at a rate at least equivalent to the oxygen uptake during respiration.

(8) The reduction of added oxaloacetate is accompanied by an equivalent oxidation of substrate.

(9) The dicarboxylic acid content (fumaric + malic + succinic + oxaloacetic) remains constant, either with or without addition of any of these acids, during respiration.

(6) and (7) are not essential because of the possibility of feeble penetration of the added acid.

If, on the other hand, fumarate is to act as a hydrogen carrier for only a part of the respiration, (2) and (5) would be essential, (8) and (9) would become a valuable addition to the evidence; but as to the rest, there would be no independent way of determining what proportion of the respiration was carried by fumarate.

For pigeon breast muscle, Annau *et al.* [1935] have produced evidence for (7) and (9). For kidney, liver and tumour none of these points has been established. The remark of Boyland and Boyland [1936] that "malignant tissue therefore seems to resemble muscle, kidney and liver in using C_4 dibasic acids as oxygen carriers" would therefore seem to be premature.

The results with brain cortex in this paper emphasise the necessity of considering the question of permeability in future work with the dicarboxylic acids.

It is to be hoped that many more facts about the effects of dicarboxylic acids on respiration will be established, so that it may be seen whether they receive their explanation from Szent-Györgyi's most stimulating theory.

SUMMARY.

The effects of malonate and fumarate on the respiration of various kinds of surviving tissue have been studied. With minced pigeon breast muscle, the effects described by Szent-Györgyi and colleagues have been confirmed. The tissue was in so damaged a condition that its respiration, even in the presence of fumarate, was strongly inhibited by physiological concentrations of calcium. With tissue which had suffered minimum damage (rat diaphragm) the effects of malonate and fumarate could still be shown. Damage by cutting this tissue facilitated the demonstration of their actions. Whether any action would occur with perfectly undamaged tissue is not clear.

Pure carbohydrate respiration (rat brain cortex and retina) was also inhibited by malonate, but fumarate only partially relieved the inhibition. Respiration (presumably carbohydrate oxidation) artificially accelerated in brain cortex and tumour by dinitro-*o*-cresol was strongly inhibited by malonate. That produced in tumour by a redox indicator was not.

These experiments were a first step in studying the applicability of Szent-Györgyi's new theory that fumarate is a hydrogen carrier in respiration. Observations on malonate inhibition cannot confirm any particular theory; but the findings do not contradict Szent-Györgyi's views, if the failure of fumarate to overcome completely the malonate inhibition in brain and retina can be ascribed to insufficient penetration of the fumarate into the tissue.

The establishing of Szent-Györgyi's theory will be very difficult. Conditions necessary for establishing a claim that the theory applies to any particular tissue are discussed.

The writer wishes to thank Prof. E. C. Dodds for the interest he has taken in this work and to express to Prof. Szent-Györgyi his appreciation of the great kindness and hospitality shown to him during his visit to Szeged.

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CXXVIII. A SIMPLE MICRO-TEST FOR ACETONE IN URINE.

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(Received March 3rd, 1936.)

THE nitroprusside test, as modified by Rothera, is commonly used for the detection of acetone bodies in urine. In practice this test has certain disadvantages. Firstly, two of the reagents, concentrated ammonia and solid or saturated ammonium sulphate, are a menace to a clinical laboratory where blood analysis is in progress, while the third reagent, sodium nitroprusside, is unstable in solution. Secondly, acetoacetic acid responds to the test much more readily than acetone, so that the response is difficult to interpret. Finally, the presence of blood or bile may mask a faint reaction.

It is well known that acetone combines with mercury salts under certain conditions giving complex precipitates. Nessler's reagent, which is potassium mercuric iodide in alkaline solution, also reacts with acetone. This test is so delicate, that if a bottle of acetone be opened near a nesslerised solution, the latter will become turbid in a few minutes. At the boiling-point, this change appears to be almost instantaneous, with the formation of a copious creamy precipitate. Under suitable conditions 0.01 mg. of acetone yields a definite precipitate with 0.5 ml. of Nessler's reagent.

Test.

Reagents: (1) Nessler's reagent [Koch and McMeekin, 1924] diluted with an equal volume of water.

(2) 5% salicylsulphonic acid in 1% sodium sulphate solution.

Apparatus: This is an apparatus previously used for the distillation of ammonia [Beaumont and Dodds, 1934]. It consists essentially of two bulbs, about 8–10 ml. in capacity, connected by a U-tube containing the liquid. When steam is blown through, the liquid oscillates in the tube, but the bulbs prevent either sucking back or blowing out. The apparatus is easily cleaned with hydrochloric acid.

Procedure: Place approximately 0.2 ml. of urine and 3 ml. of acid in an 8 × 1 in. monax test-tube. Add a porcelain chip. Close the tube with the trap tube containing 0.5 ml. of diluted Nessler's reagent. Place the tube on a sand-bath and heat steadily and strongly until steam begins to pass through the Nessler's reagent. If the sand-bath is hot, this process takes 1–2 min.

Interpretation.

The test is given by acetone and acetoacetic acid since the latter is decomposed by boiling in acid solution. Under the above conditions normal urines give no definite response. A small but definite creamy precipitate is given by acetone solutions containing 0.01 mg. per ml. When urine contains more than 1%

acetone, the reaction is characteristic. The dense initial precipitate, which appears, dissolves gradually and may be reprecipitated by continued boiling. Volatile reducing substances, *e.g.* formaldehyde, when present in urine, interfere with the test, giving a red precipitate. In this case, repeat the test, adding one drop of 1 % sodium hypochlorite to the Nessler's reagent before heating. This alteration renders the test less sensitive. The normal output of acetone and acetoacetic acid is generally considered to be less than 20 mg. *per diem* or 0.01 mg. per ml. The test may also be applied to blood filtrates; in this case 2 ml. tungstic acid filtrate are used and treated as described above.

I wish to express my thanks to Prof. E. C. Dodds for his kindness in allowing me to carry out this work.

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CXXIX. THE QUANTITATIVE DETERMINATION OF VITAMIN K. I.

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(Received March 30, 1936.)

IN a recent paper [Dam and Schönheyder, 1934] a deficiency disease in chicks was described, the main symptoms of which are a tendency to large haemorrhages, certain pathological changes in the gizzard and anaemia. The blood of chicks suffering from this disease has a considerably prolonged clotting time. The cause of the disease was ascribed by Dam [1935, 1, 2] to lack of a specific fat-soluble vitamin which is called vitamin K. Ahnquist and Stokstad [1935], working independently of Dam, have already confirmed this suggestion. The delayed clotting was occasionally observed by Dam [1930] and by McFarlane *et al.* [1931] and has recently been subjected to more intensive study by Schönheyder [1935; 1936].

Out of a total of 323 animals presenting symptoms of vitamin K deficiency, prolonged coagulation time (over 10 min.) was found in 86.5%. The clotting time was determined in the following manner. The brachial vein was opened by a slight cut and 2-3 ml. of blood were allowed to drop into a small porcelain bowl. 30-45 sec. were usually required to obtain this amount of blood. The period of time from the venous puncture until complete clotting is called the clotting time. This is between 1 and 10 min. in normal chicks, while it may be several hours for diseased animals. This simple procedure enables one to differentiate between normal and diseased animals, and the results are in accordance with those obtained by a more exact method of measuring the clotting time (see later).

Previous research [Schönheyder, 1936] on the subject has shown that the prolonged clotting time cannot be due to insufficiency of thrombokinase in the tissue or thrombocytes, or to insufficiency of fibrinogen in the plasma or an accumulation of anticoagulating agents, especially antiprothrombin, in the plasma. The serum calcium values were usually found low and the inorganic phosphorus values constantly elevated in the diseased animals. The addition of Ca^{++} to the plasma of the diseased animals did not shorten the clotting time, and no change in p_{H} or salt content of plasma could be demonstrated.

It was shown, however, that per unit of time and under identical conditions far less thrombin was formed, after addition of thrombokinase, in abnormal plasma than in normal. Normal plasma was further found to contain a surplus of a certain ingredient which accelerates the coagulation process in chickens having lack of vitamin K. The most probable explanation of the prolonged clotting time in the diseased animals is that it is due to reduced concentration of prothrombin.

As with other vitamins, two methods of biological assay, preventive and curative, may also be used for vitamin K. By means of the preventive method we have found that a portion of dried pig's liver equivalent to 20% of the diet gave complete protection while 10% afforded only partial protection. All portions of pig's liver are not of equal potency.

The curative method.

It appeared, that by giving the animals with a considerably prolonged clotting time a vitamin K test substance, it was possible to make the clotting time normal. The animals suffering from vitamin K deficiency became normal with reference to clotting time in 3 days when given sufficient food containing vitamin K. The administration of 11.3 mg. of dried pig's liver per g. chick daily for 3 days was found sufficient to restore the coagulation time to normal values. The curative method of assaying vitamin K is based on this fact. Meanwhile it appeared desirable to find a more exact method of measuring the clotting time. The method of Fischer [1930] appeared to be most suitable as it is especially founded on experiments made with plasma from hens. It is well known that blood from hens does not clot when it is shed through a cannula which is inserted in a blood vessel. In this manner it is possible to obtain blood under identical circumstances. The plasma thus obtained does not clot until a clotting agent is added. Normal and diseased plasmata show an enormous difference in clotting time in the presence of the same clotting agent. Instead of using a clotting agent of constant strength, the concentration of the clotting agent is increased until the plasma of the K-avitaminous animal is made to clot as quickly as normal plasma. The relation between the concentrations of the clotting agent which clot a plasma from a sick animal and normal plasma in the same time indicates the degree of vitamin K deficiency. For practical reasons we have chosen to multiply this relation by 10. This figure, which expresses the animal's condition, is called the *S* figure of the animal (*S*=degree of sickness). If *S* is equal to or less than 10 the animal is normal as regards vitamin K. *S*₁ and *S*₂ are taken to be the numerical expression of the animal's condition previous to the vitamin assaying and after the test substance has been given for 3 days respectively. One unit of vitamin K is defined as the least amount of a test substance per day per g. of chick which is able to reduce the animal's *S*₁=1500–2000 to *S*₂=10 when given for 3 days. This unit is small but when it is multiplied by 1000 it equals the amount of substance which is necessary to cure an animal with *S*₁=1500–2000 and a weight of 333 g. in the course of 3 days, the animal receiving one-third every day.

The basal diet is a modification of diet 124 of Dam [1935. 2].

Caseinogen	10
Dried pig's liver extracted with ether	10
Dried yeast	15
Sucrose	62.3
Salts No. 2	2.7
						<hr/> 100
Cod liver oil	4

The caseinogen preparation used was free from vitamin A. It was identical with the preparation used at Statens Vitamin-Laboratorium. According to the declaration of the manufacturer (Dansk Mejeri Industri and Exportkompagni, Copenhagen), it consists of lactic acid caseinogen, which has been subjected to drying in air at room temperature for 24 hours. The liver is spread on thin trays and dried in a current of air not over 40°. The dried liver is scraped from the trays and ground to a fine powder. This is first extracted with light petroleum, which dissolves most of the fat. The rest dissolves when extracted with ether in Soxhlet's apparatus, leaving a fat-free residue. The yeast, which is first autolysed for a day at 40° and then spread out on trays to dry at 40°, is ground to a fine powder. Ordinary grade sugar is used. Salts No. 2, CaCO₃ 2.00, MgCO₃ 0.10; ferric citrate 0.16; NaCl 0.44; KI 0.000025. When these ingredients have been mixed 4 % fresh cod liver oil (from Peder Møller, Oslo) is added.

The animals all became equally ill whether they had received this food from the time of hatching or from the age of 2 weeks. During the first 2 weeks the animals therefore were given Oluf Nielsen's chicken food. The diet was then changed. The animals must be kept on the basal diet mentioned for at least 3 weeks, *i.e.* until 35 days old. Experience has taught us the important fact that when chicks of the same breed are given identically prepared diets there is very little variation in the severity of the attack (S_1) and that this is independent of the age (30–60 days) and the weight during this period. The decisive factor when determining S is the animal's weight. Not until this is 200 g. is it possible to obtain blood under standard conditions. Since haemorrhages occur more frequently the more animals there are in a cage because of mechanical injuries it is advisable to have as few as possible together.

The test substance should be given as tablets. If a small quantity of a substance is to be measured the substance is dissolved in a few ml. of an indifferent oil, for example olive oil. The olive oil is then mixed with a suitable quantity (25 g.) of the basal diet and the whole mixture is made into tablets.

Blood samples. Carel's method [1922] for adult hens is used.

The blood is not taken aseptically. An assistant holds the chick so that it lies on its back on a low table, the animal's neck being allowed to hang over the edge and its head being bent so far back that the front of the throat forms a convex surface. Anaesthesia is not necessary since the animal apparently goes into a hypnotic state in this position. An incision 1.5 cm. long is now made in the mid-line between the base of the head and the upper breast aperture. The skin and subcutis are drawn aside and the muscles and the anterior surface of the columna are now seen, covered with a thin fascia, which is incised. It is easy to find the two carotid arteries which lie close together apparently in the same sheath; they are separated from one another with forceps and one of the arteries is exposed in the field of operation by means of a retractor, and the vessel is held in place by means of a ligature with long ends. A small artery clamp is applied 1 cm. proximal to the ligature on the vessel. The whole operative area is rinsed with Ringer's solution, followed by mineral oil. With a sharp pair of scissors a small incision is now made between the ligature and the artery clamp and the cannula is inserted into the lumen of the blood vessel. The clamp is taken off and the blood allowed to flow through the cannula and collected in an ice-cooled paraffin-lined glass. From 3 to 4 ml. are taken. After centrifuging the plasma is pipetted off and is now ready for use in the clotting experiment.

Determination of clotting time. Since plasma, when clotting agent of constant strength is added, coagulates more and more slowly for each day of standing, it is necessary to undertake the clotting determination on the day of operation. The plasma is always diluted with an equal volume of Ringer's solution, thereby making several determinations possible. One drop of clotting agent is invariably added to 5 drops of diluted plasma. The method of Fischer [1930] was used without modification for determination of the clotting time.

The measurement takes place in a water thermostat at 40°. 5 drops of diluted plasma are dropped into a Fischer's miniature test-tube (30 × 8 mm.; thickness 0.9 mm.). The test-tube is placed in a metal stand, *i.e.* a holder especially constructed to accommodate 15 miniature test-tubes in a row. It can be opened and closed as the upper wall is on a hinge at one end and can be tightened at the other with a screwing device. The underside of the movable wall is covered with a thin layer of rubber which fits tightly over the opening of the tubes. A thin layer of parchment paper is always placed over the rubber, forming a covering above the test-tubes. This must be changed between every determination in order to prevent impurities being conveyed from experiment to experiment.

The holder is closed at once after the clotting agent has been added to the plasma in the glasses and is then transferred to the thermostat. It is inverted once, to mix the contents of the tubes,

and at this moment a stopwatch is started. At the moment the plasma is no longer liquid the time is noticed. The determinations are made in duplicate. We generally work with clotting times between 120 and 600 sec.

A watery extract of hens' lungs was used as clotting agent. Ethyl green was added as antiseptic. The lung extract must be kept in a refrigerator and retains its properties for some time. It is constantly necessary to test the clotting agent on normal animals. No difference whatsoever could be shown in the clotting times in normal adult hens and normal chicks, measuring with the same clotting agent. When the clotting agent is to be used for a determination we make several dilutions with Ringer's solution. The lung extract must be shaken before use. It is necessary to wait 15 min. before using the various dilutions, as they require this period to obtain a constant strength which then remains unchanged for 3-4 hours. The plasma to be examined should be kept on ice. The pipettes are placed in distilled water.

Calculation of the S-values. Fischer [1935] has recently shown that the following equation applies to the relation between the clotting time and the concentration of the clotting agent: $\frac{1}{t} = KC^a$, where t = clotting time, c = concentration of clotting agent, and K and a are constants. Using logarithms in the above equation we get: $\log \frac{1}{t} = \log K + a \log C = K_1 + a \log C$.

This equation is merely an approximation to the actual relationship, but for practical use it is obvious that interpolation is facilitated by obtaining a straight line. The above-mentioned logarithmic dependence was found to deviate only slightly from a straight line and was therefore suitable for the purpose. If a curve has been plotted for the related values of log conc. and $\log \frac{1000}{t}$, it is only necessary to interpolate to the log conc., which corresponds to

$$\log \frac{1000}{t} = \log \frac{1000}{180} = 0.74.$$

The following is an example of a test of the clotting agent and the measurement of S in an arbitrarily chosen plasma.

(1) Plasma from a normal chick.

Lung extract in the added drop %	log conc.	$\log \frac{1000}{t}$
0.125	0.0097	0.469
0.25	0.0398	0.602
0.50	0.0699	0.762
0.75	0.0875	0.854
1.00	0.1	0.886

The points are plotted with the abscissa giving the log conc. and the ordinate $\log \frac{1000}{t}$. A curve is drawn between the points and its point of intersection with

(2) Plasma from chick No. 1298.

Lung extract in the added drop %	log conc.	$\log \frac{1000}{t}$
1.25	0.097	0.057
2.50	0.398	0.222
5.00	0.699	0.392
10.00	1.000	0.619
15.00	1.176	0.762
20.00	1.301	0.824
33.33	1.522	0.903
50.00	1.699	0.921
75.00	1.875	0.947

a line drawn parallel with the abscissa through 0.74 on the ordinate axis is determined. The point of intersection corresponds to 0.066 on the abscissa. This means that 0.46 % lung extract can bring about clotting of normal plasma in 180 sec.

The data are treated as before. The point of intersection corresponds to 1.16 on the abscissa. Antilog $1.16 = 14.46$. According to this the S value of the chick will be $\frac{14.46 \times 10}{0.46} = 314$.

A provisional curve for the relationship between the concentration of the substance and its effect. During the whole experiment the same portion of dried pig's liver was used. It was given in tablets of 2.5 g. each. Before receiving the tablets all animals are assumed to have had $S_1 = 1500$.

Chicken No.	mg. dried liver per g. chick per day for 3 days	S_2	Chicken No.	mg. dried liver per g. chick per day for 3 days	S_2
1260	1.6	500	1312	6.8	40
1275	2.2	162	1184	7.2	21
1292	2.8	178	1265	8.2	19
1293	3.0	136	1286	9.9	12
1195	3.7	78	1214	10.2	10
1268	4.4	47	1194	11.3	9
1297	4.8	62	1217	14.8	8
1289	6.1	39	1279	17.8	9

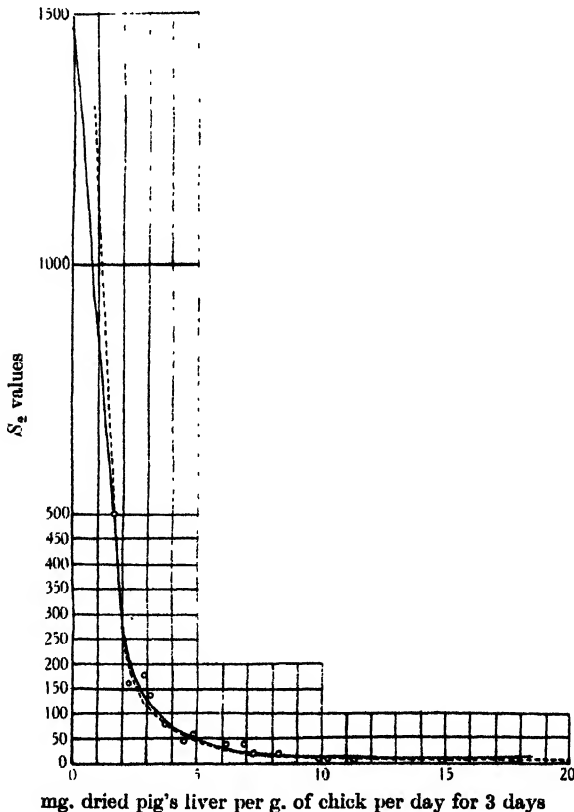


Fig. 1.

We see that about 10 mg. of dried pig's liver per g. of chick per day are the least quantity which is able to reduce $S_1 = ca. 1500$ to $S_2 = 10$. About 10 mg. dried liver contain one vitamin K unit.

The curve shows that as far as exact results are concerned it is not very important to have a specific S_1 at the beginning of the measurement, provided it is more than 1500. For example we see that in order to reduce $S_1 = 500$ to $S_2 = 10$, 8.5 mg. of dried liver per g. per day are necessary. To reduce $S_1 = 1000$ to $S_2 = 10$, 9.3 mg. are required, i.e. a difference of 10 %. Therefore if it is certain, at the beginning of the experiment, that S is greater than 1000 the percentage error cannot exceed 10 % in this respect, if enough test substance is given almost to cure the animals.

As seen above, the curve resembles a hyperbola. The author is inclined to believe that the relationship between the S -values and the amount of the test substance in mg. may be expressed as follows:

$$S_2 \cdot \text{mg.}^2 = 1100.$$

The dotted curve is plotted from this equation. The curve is used as follows. The dose of test substance must be so chosen that S_2 falls to the left of the point of intersection between the curve and the horizontal line through $S_2 = 10$ on the ordinate parallel with the axis of abscissae. Thus it is seen that if a certain amount of a substance per g. of chick per day for 3 days reduces $S_1 = 1500$ to $S_2 = 50$, we may conclude that the quantity of substance in question contains $1/2$ vitamin unit. Twice the amount would be the least quantity which is able to make $S_2 = 10$.

The number of animals required for an exact determination of the curve depends upon the exactitude demanded when determining a vitamin K unit. It might be desirable to determine the curve with other sources of vitamin K than the pig's liver used here. At present we believe that the curve represents general conditions, even though a number of supplementary studies should be made in the future. A more exact evaluation of the technique as well as a mathematical application of the curve based on more extensive data will be reported later.

SUMMARY.

Vitamin K is best estimated by the curative method. An animal suffering from vitamin K deficiency becomes normal with reference to clotting time in 3 days, when food containing sufficient vitamin K is given. It was decided to characterise a plasma by the concentration of clotting agent which upon the addition of 1 drop to 5 drops of 50 % plasma would cause the latter to clot in 180 sec. at 40°.

The relation between the concentration which is necessary to clot a diseased plasma in 180 sec. and that which is necessary to clot a normal plasma presents a quantitative expression of the degree of sickness of the animal. The relation multiplied by 10 is called the animal's S value. A vitamin K unit is defined as the smallest daily dose of a test substance per g. of chicken given for 3 days which is able to reduce the S value from over 1500 to 10. The substance to be tested for vitamin K should be given in tablet form.

The methods of drawing blood from the carotid artery, of determining the coagulation time and of calculating the S value are described. A provisional curve of standardisation is given.

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CXXX. THE OCCURRENCE AND CHEMICAL NATURE OF VITAMIN K.

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(Received March 27th, 1936.)

It has been shown that the antihaemorrhagic factor of the chick is a fat-soluble and rather thermostable vitamin which can be distinguished from vitamins A, D and E [Dam, 1935, 1, 2; Schönheyder, 1935; Almquist and Stokstad, 1935, 1, 2]. One of the sources of this vitamin—for which the letter K has been proposed [Dam, 1935, 1, 2]—is hog liver fat, where it may be found in the easily soluble non-sterol fraction of the non-saponifiable matter, when cold saponification is used. On partitioning between light petroleum and 90 % methyl alcohol the vitamin was found in the light petroleum layer [Dam, 1935, 1, 2]. Its occurrence in hog liver fat, hemp seed *etc.* was established by means of the preventive method.

Since then a curative and more convenient method has been published by Schönheyder [1936] and it has thereby been possible to determine the content of vitamin K in different foodstuffs with some degree of accuracy and to gain further information about the chemical properties, which are to be taken into account during attempts to isolate the substance.

The method used in the assay was that described by Schönheyder [1936]. The substances were given on 3 successive days in the form of weighed tablets, either alone or, when fats and oils were to be tested, in mixture with a certain amount of the basal diet, one or two animals being used for each determination.

The unit is that amount of the vitamin which is required per g. of the animal on 3 successive days in order to render the clotting power of the blood normal (the whole treatment of an animal weighing 333 g. will require 1000 units).

The simple chemical treatments used in connection with the work will appear from the tables and description.

Table I.

	Units per g.
Dried cabbage, white*	230
Dried cabbage, red*	60
Dried cabbage, "Spitzkohl"*	240
Dried kale*	260
Dried spinach*	270
Dried spinach, "selected quality"†	540
Dried alfalfa, Producer I†	136
Dried alfalfa, Producer II†	250
Fresh carrot, calculated on dry matter	190
Fresh potato, calculated on dry matter	20
Barley	15
Dried hog liver‡	100
Dried dog liver§	67
Dried chicken liver, normal§	< 11
Dried chicken liver, K-avitaminous§	< 11
Dried cod liver§	10

* Dried in air 60°.

† Dried commercially in air of high temperature for a very short time.

‡ Dried in air 40°.

§ Dried in a vacuum desiccator at room temperature.

1. Occurrence of vitamin K in different plant materials and livers.

The figures in Table I do not pretend to represent mean values but are merely obtained by means of 1 or 2 samples of each substance. They do, however, give a very valuable orientation.

The high activity of vegetables is obvious, and the statement of Almquist and Stockstad that alfalfa is an active material is confirmed by these as well as by our previous findings.

It is perhaps somewhat surprising that the liver of normal chicks is a rather poor source of vitamin K.

2. General chemical properties of vitamin K in hog liver fat.

Hog liver fat was, as mentioned before, one of the first sources used in our early investigations on vitamin K. Table II shows the result of different chemical treatments of this substance.

Table II.

	Units calculated per g. liver fat	Loss by saponifi- cation %
Hog liver fat procured 4. vi. 35:		
Without any treatment	266	288
Without any treatment	309	
Non-saponifiable matter (cold saponification 5 min. with the calculated amount of KOH in methyl alcohol)	107	63
(a) Fatty acid fraction	0	—
Fatty acid fraction	0	—
Water-soluble fraction obtained by the saponification	0	—
Liver fat after removal of the free fatty acids	307	—
Hog liver fat procured 16. iii. 35:		
Without any treatment	490	—
(b) Non-saponifiable matter freed from cholesterol by crystallisation from light petroleum (cold saponification 6 hours with 100% excess of KOH in methyl alcohol)	139	129
The same	119	
Non-saponifiable matter (b) again treated with the same amount of the saponification agent at room temperature for 45 hours	93	28
Non-saponifiable matter (b) treated with the saponification agent on the boiling water-bath for 6 hours	0	100
Non-saponifiable matter (b) + fatty acids (a)	370	—
Non-saponifiable matter (b) + fatty acids (a)	400	—
Non-saponifiable matter (b) heated at 100°, 13 hours	143	—
Non-saponifiable matter (b) + ether containing peroxide, 23 hours	101	—
Liver fat after standing in light and air 147 days	143	—

During work with the preventive method we noted that the process of cold saponification involved a loss in vitamin K activity. This observation was fully confirmed by the curative technique, not only for hog liver fat but also for plant extracts (the saponification experiments with the latter are not reported in this paper in order to avoid unnecessary repetition). It appears from the table that cold saponification lessens the activity about 60–70%, the duration of the treatment and the excess of KOH being of relatively small influence, while hot saponification destroys the activity completely. The fatty acid fraction (obtained by ether extraction at acid reaction after complete removal of the non-saponifiable matter at alkaline reaction) was found to be inactive in accordance with previous observations by means of the preventive method. The water-soluble

fraction (containing glycerol, glycerophosphoric acid *etc.* and eventually unknown compounds) was also, after neutralisation and precipitation of inorganic salts by alcohol, found to be inactive.

When the fatty acids were added to the non-saponifiable matter, in the same proportion as in the liver fat, the activity was to a large extent restored. The significance of this interesting result is being investigated further. Removal of the free fatty acids of the liver fat had no influence (hog liver fat contains much free acid, the sample from 4. vi. 35 had a saponification value of 160.5 and an acid value of 79.5).

Heating at 100° for 13 hours did not diminish the activity of the non-saponifiable matter. This finding is in accordance with our previous results with liver fat and with the results of Almquist and Stokstad.

Ether containing peroxide (giving a black precipitate with metallic mercury) did not, within 23 hours, effect any large destruction of the vitamin in the non-saponifiable matter, but keeping the liver fat in light and air in the laboratory for 147 days diminished the activity of the liver fat to 29 % of the original value. Whether this is due to the direct action of the light or to rancidity has not yet been elucidated.

Channon *et al.* [1934] have isolated a new hydrocarbon from hog liver fat ($C_{43}H_{76}$ or $C_{50}H_{84}$) which has about the same solubility properties with regard to light petroleum and methyl alcohol as vitamin K. A sample of this substance, courteously furnished by Prof. Channon, was tested for vitamin K; 17 mg. were completely inactive when given to a chick weighing 400 g.

3. Extraction of the vitamin from vegetables.

Previous experiments with hemp seed disclosed the fact that it is difficult to extract the vitamin from this material completely with ether. We have therefore now tested the effect of different solvents on a green vegetable, *viz.* a sample of dried alfalfa. The extraction was carried out in a Soxhlet apparatus surrounded with a vapour jacket whereby the solvent was kept at the boiling point while in contact with the substance to be extracted (see Table III).

Table III. *Extraction of dried alfalfa (138 units per g.) at the boiling point of the solvent.*

Solvent	Duration of extraction hours	Extract % of dried alfalfa	Units per g. extract	Units in the extract of 1 g. dried alfalfa	Apparent output %
Ether	50	3.5	3900	136	100
Alcohol 99 %	10	5.37*	5000*	268*	197
Acetone	3.5	7.15	4160	296	218
CCl_4	13	4.35	4160	174	128

* Calculated on the ether-soluble portion of the alcoholic extract.

It is obvious that a prolonged treatment with ether would appear to extract all the vitamin of the sample (138 units per g.). But the results with the other solvents show that it is possible to obtain a somewhat larger quantity of the vitamin in the extract than that which was found by direct measurement of the dried green vegetable. This effect, which has been established in a series of experiments (not described in this paper) is most probably to be explained by assuming that the animal alimentary tract cannot completely extract vitamin K from dried green vegetables while a much better extraction may be obtained by means of suitable solvents. Acetone appears to be particularly suited for the purpose.

When referring to the content of vitamin K in a sample of dried green vegetable it is, therefore, necessary to distinguish between the effective value, found by feeding the vegetable directly, and the absolute content found after suitable extraction.

4. *Further concentration of the vitamin.*

During the work on liver fat with the preventive technique, it was found that the vitamin remains in the light petroleum layer when the petroleum solution is shaken repeatedly with 90 % methyl alcohol. Almquist and Stokstad, however, state that they found the light petroleum and the methyl alcohol fractions equally active. By means of the present method we have found that when the light petroleum-soluble portion of an acetone extract from alfalfa containing 11,000 units per g. was shaken with 2/3 of its volume of 90 % methyl alcohol three times, the petroleum fraction, representing 39 % of the whole, had 25,000 units per g. while the methyl alcohol fraction had less than 250 units per g. After separation of a less active fraction from absolute alcohol by leaving it in the ice box for several days, and repetition of the partitioning between light petroleum and 90 % methyl alcohol it was possible to obtain a preparation containing 190,000 units per g.

By adsorption on Al_2O_3 and subsequent elution with benzene-alcohol it was possible to recover 87 % of the dry matter from this material, and this fraction (filtrate + eluate) could be shown to be practically inactive. Al_2O_3 , apparently, holds the vitamin (and certain of the chlorophyll decomposition products) so firmly that elution is practically impossible in the ordinary way. This experiment shows that, assuming that the vitamin is not destroyed by the adsorption on Al_2O_3 , the pure vitamin must contain more than $2 \cdot 10^6$ units per g.

When working with small quantities, it was possible to adsorb the vitamin on CaCO_3 or cane sugar from light petroleum. When the pigments including chlorophyll had passed through the column, the vitamin could be eluted by methyl alcohol (or by dissolving the sugar in water and shaking with light petroleum). In this way about the half of the vitamin could be obtained, from CaCO_3 , in a concentration of 600,000 units per g. while the treatment with cane sugar yielded about one-third in a concentration of 10^6 units per g. This latter preparation is a viscid oil. It is the strongest concentrate hitherto prepared. 1 mg. is sufficient to render the clotting of the blood of a 333 g. chick normal.

The concentration and adsorption methods are at present being elaborated with a view to large scale operation and final isolation of the substance.

The concentration process.

2.15 kg. of dried alfalfa (effective content 250 units per g.) were extracted with acetone in a Soxhlet apparatus for 21 hours. The acetone was distilled off and the residue was repeatedly agitated with light petroleum and filtered. Weight of light petroleum extract 70 g. with 11,000 units per g.

The light petroleum solution, 1270 ml., was treated with 810 ml. methyl alcohol and 90 ml. water 3 times in a tap-funnel.

Light petroleum fraction 27 g. with 25,000 units per g.

Methyl alcohol fraction 43 g. with < 250 units per g.

The light petroleum fraction was taken to dryness, dissolved in absolute alcohol and placed in the ice-box for several days. After filtration the dissolved portion contained about 50,000 units per g. After repeated shaking with 90 % methyl alcohol, the light petroleum-soluble fraction was brought up to a strength of 190,000 units per g.

Adsorption on Al_2O_3 (Merck puriss). 2.3 g. substance (440,000 units) in 50 ml. light petroleum were passed through a 5×30 cm. column of the adsorbent, washed with light petroleum and a mixture of this and benzene (30 + 70), elution with benzene-ethyl alcohol (50 + 50):

Filtrate: 0.2 g. (crystals resembling hentriacontane).

Combined eluate: 1.8 g. (including carotene zones and a green-brown zone with subdivisions).

Filtrate and combined eluate contained < 20,000 units per g. < 40,000 units in all.

Adsorption on finely powdered cane sugar. 1 ml. of a solution of the concentrate in light petroleum containing 56 mg. = 10,600 units, was passed through a 20×20 mm. column of the adsorbent and washed with light petroleum until the pigment zones had just passed through the column. The adsorbed substance was then eluted by dissolving the sugar in water and shaking the solution with light petroleum several times.

Eluate: 3.6 mg. with 10^6 units per g. = 3600 units. Filtrate: 50.4 mg. with 60,000 units per g. = 3000 units.

Adsorption on $CaCO_3$ (Merck pro analysi). 1 ml. of the same solution was passed through a 15×22 mm. column of the adsorbent and treated in the same way. The elution was effected by methyl alcohol-acetone (equal parts):

Eluate: 8.8 mg. with 600,000 units per g. = 5300 units. Filtrate: 50.8 mg. with 60,000 units per g. = 3000 units.

SUMMARY.

1. A series of substances has been tested quantitatively with respect to their content of vitamin K. Green vegetables are particularly rich sources of the vitamin.

2. Cold saponification of hog liver fat lessens the activity to about one-third of the original value and hot saponification destroys it completely. The fatty acid fraction is inactive *per se* but appears to enhance the activity of the non-saponifiable matter obtained by cold saponification. The thermostability of the vitamin is confirmed.

3. The efficiency of different solvents in extracting vitamin K from dried alfalfa has been studied. Acetone has been found to be particularly suited for the purpose. Certain solvents (alcohol, acetone) extract more vitamin K from dried alfalfa than could be found by directly feeding the vegetable. This is explained by assuming that the extraction in the alimentary tract is not complete.

4. Attempts to concentrate the vitamin from dried alfalfa have been made. The method of removing inactive material from a light petroleum solution of the extract by means of 90% methyl alcohol has given good results. Al_2O_3 adsorbs the vitamin so firmly that it cannot be eluted by ethyl alcohol-benzene, but it is possible to obtain a concentration up to 600,000–1,000,000 units per g. by means of $CaCO_3$ or cane sugar.

Part of this work was aided by a grant from P. Carl Petersens Fond.

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CXXXI. A NEW IODIMETRIC PROCEDURE FOR THE ESTIMATION OF CHLORIDE IN SMALL AMOUNTS OF BLOOD.

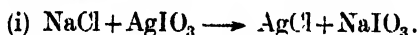
BY GEOFFREY ARTHUR DERING HASLEWOOD AND
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(Received April 1st, 1936.)

THE commoner methods for the estimation of blood chloride are modifications of the Volhard titration [*e.g.* Whitehorn, 1921; Van Slyke, 1923]. In the micro-modifications [Smirk, 1927; Patterson, 1928] the use of solvents such as alcohol or acetone is necessary to obtain even semi-permanent end-points. The possibility of chloride loss in the "open" Carius acid digestion of the blood prior to chloride estimation has frequently been pointed out [*vide* Norris and Ampt, 1933]. The iodimetric procedure of McLean and Van Slyke [1915] has been applied to Folin-Wu blood filtrates by Gettler [1921], Hanna [1928] and others, but, as Hanna remarks, is not readily adaptable to small amounts of blood. Use of adsorption indicators has been recommended [Saifer and Kornblum, 1935]. The above procedures are essentially refinements of macro-methods and are not "micro" in the same sense as the procedure to be described, where six atoms of analysable constituent (iodine) are available for titration for each chloride ion originally present.

The method here described is based on the reaction:



Silver iodate is added in ammoniacal solution to the chloride solutions, and with the silver chloride formed, is precipitated by excess of acid. After filtration, iodine is liberated from the soluble iodate formed and is titrated with 0.005*N* sodium thiosulphate. Van Slyke *et al.* [1927] estimated sulphate in a somewhat analogous manner by the addition of solid barium iodate to the sulphate solution, followed by gasometric determination of the liberated iodate.

The solubility of silver iodate in water is approximately 4 mg./100 ml. at 25°, while the figure for silver chloride is approximately 0.15 mg./100 ml. There is therefore a "blank" titration due to dissolved silver iodate.

This "blank" will depend on the equilibria:



Excess of soluble iodate, added or set free according to (i), will depress the "blank" due to dissolved silver iodate.

Chloride-free mixtures, containing silver iodate, were treated with amounts of potassium iodate, calculated from (i), corresponding to various concentrations of chloride. The "blanks" (*i.e.* the titration of the additional iodate found) were added to the "theoretical" titrations, also calculated from (i). The figures thus obtained agreed almost exactly with the experimentally found titrations with chloride solutions of corresponding concentrations. This fact shows that (i), for practical purposes, goes to completion (see Table I).

The reaction was studied over the range 0–80 mg. of sodium chloride per 100 ml. It was found that the titration of iodate set free from sodium chloride solutions of concentrations from 10 to 40 mg./100 ml. had to the concentration a relationship which could be expressed by an equation of the form $\text{NaCl (mg./100 ml.)} = a (\text{titration} - b)$. From 40 to 80 mg. NaCl/100 ml., the iodate liberated corresponded almost exactly with (i); i.e. the "blank" could be neglected and the chloride calculated directly from (i).

The method gave identical results with sodium, potassium, calcium and magnesium chlorides. For blood or plasma, it was found convenient to use the Somogyi [1930] zinc hydroxide method of protein removal. Sodium chloride added to such a filtrate, previously freed from chloride (with silver nitrate, followed by hydrogen sulphide) was quantitatively recovered. Good recovery of sodium chloride added to blood was also obtained. Analytical results on 0.2 ml. of whole blood or plasma agreed within 1 % among themselves and with gravimetric analyses carried out on larger samples of the original blood or plasma.

EXPERIMENTAL.

Preparation of silver iodate. Solid silver iodate was prepared from silver nitrate (in very slight excess) and potassium iodate. 2 g. of the washed and dried solid were dissolved in 100 ml. *N* ammonia. Both solid silver iodate and its ammoniacal solution appear to decompose slightly on keeping with liberation of soluble iodate. Immediately before a series of determinations, therefore, 5 ml. of the "stock" 2 % ammoniacal silver iodate were acidified with 2 *N* sulphuric acid (5 ml.) and centrifuged. After removal of the supernatant liquid, the iodate was redissolved in fresh *N* ammonia (5 ml.). This "silver iodate reagent" lasted unchanged at least 1 day. It was not improved by preliminary washing of the precipitated silver iodate.

Technique of the analyses. The chloride solution (2 ml.) was treated with 1 ml. of the above silver iodate reagent and, after careful mixing, with 1 ml. of 2 *N* sulphuric acid. The mixture was shaken and filtered through a small fine paper. 2 ml. of the filtrate were treated with potassium iodide (1 ml. of 1 %) and the liberated iodine was titrated with 0.005 *N* sodium thiosulphate, with starch as indicator. Such titrations, using 2 ml. of sodium chloride solutions of concentrations from 0 to 80 mg./100 ml., are shown in Table I, col. 4.

Table I.

Chloride concentration (mg. NaCl/100 ml.)	Theoretical titre	Silver iodate "blank"	Experimental titre
	ml. of 0.005 <i>N</i> sodium thiosulphate		
0	0	1.25	—
5	1.03	0.65	1.75
10	2.05	0.51	2.60
15	3.08	0.46	3.57
20	4.10	0.37	4.50
25	5.13	0.30	5.42
30	6.16	0.20	6.37
35	7.19	0.14	7.31
40	8.20	0.10	8.25
45	9.23	—	9.24
50	10.26	—	10.20
60	12.30	—	12.30
70	14.35	—	14.34
80	16.41	—	16.30

Determination of silver iodate "blanks". The quantities of iodate, as 0.1 *N* potassium iodate, which should be set free according to (i) from the different chloride solutions used, were made up with water to 2 ml. The resulting solution, in each case, was treated exactly as the chloride solution in the above procedure. The experiment was then repeated, but with addition of *N* ammonia (1 ml.) instead of the silver iodate reagent. The difference between the titration figures gave the silver iodate "blank" for the particular chloride concentration. The "blanks" are shown in Table 1, col. 3.

Calculation of results. In Fig. 1 the concentration of sodium chloride has been plotted against the "theoretical" and experimental titrations of 0.005 *N* sodium thiosulphate. Above 40 mg. NaCl/100 ml. the two lines are identical; below 40 mg./100 ml. the experimental curve deviates somewhat from the theoretical, but between 15 and 40 mg. (curve B) it is practically a straight line. The equation of curve B is

$$\text{NaCl (mg./100 ml.)} = 5.275 (\text{titre} - 0.65),$$

and that of the theoretical curve, A, is

$$\text{NaCl (mg./100 ml.)} = 4.875 \times \text{titre}.$$

Hence, at the point of junction, *P*,

$$5.275 (\text{titre} - 0.65) = 4.875 \times \text{titre}.$$

Hence

$$\text{titre} = 8.575 \text{ ml.}$$

And

$$\text{NaCl} = 41.8 \text{ mg./100 ml.}$$

From 15 to 40 mg./100 ml., therefore, the formula corresponding to curve B is applicable, while from 40 to 80 mg./100 ml. the equation of curve A is used.

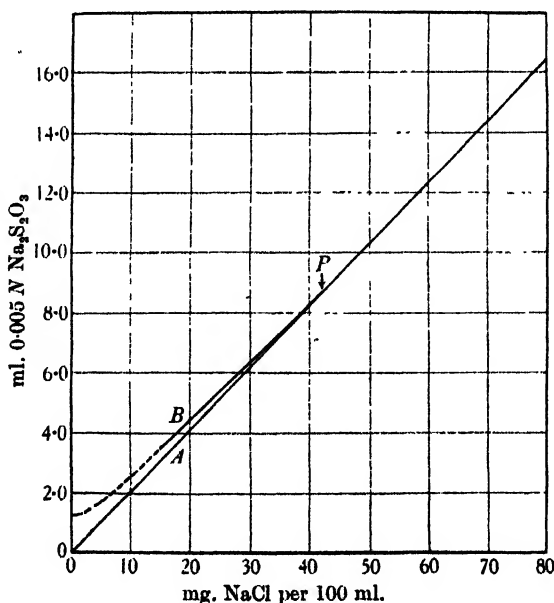


Fig. 1.

Application to blood. 2 ml. of a Somogyi zinc hydroxide filtrate (1 in 10 dilution) are used exactly as in the case of the chloride solutions above.

On the small scale the method is as follows: 0.2 ml. of whole blood (or plasma) is pipetted into 1.4 ml. of water (1.0 ml. for plasma). 0.2 ml. of 10 % zinc sulphate and 0.2 ml. of 0.5 *N* NaOH (0.4 ml. of each reagent for plasma) are added and thoroughly mixed. The mixtures are then centrifuged. 1.0 ml. of the supernatant liquid (\approx 0.1 ml. of blood or plasma) is treated with silver iodate reagent (0.5 ml.) and, after mixing, with 2 *N* sulphuric acid (0.5 ml.). The mixture is shaken and filtered through a small fine paper. 1.0 ml. of filtrate (\approx 0.05 ml. of blood or plasma) with the addition of potassium iodide (1.0 ml. of 1 %), is titrated with 0.005 *N* sodium thiosulphate with starch as indicator.

Calculation of results. Chloride (mg. NaCl/100 ml. of blood or plasma = $97.5 \times \text{titre}$).

If the titre is less than $8.575/2$, i.e. 4.30 ml., curve B must be used, and the calculation becomes: chloride (mg. NaCl/100 ml. of blood or plasma = $105.5 (\text{titre} - 0.65)$).

Comparison of results. NaCl (mg./100 ml.); plasma A: gravimetric, 675; new method, 672. Plasma B: gravimetric, 612, 614; new method, 614. Blood B: gravimetric, 422, 414; new method, 423.

SUMMARY.

A new method for the estimation of chloride is described, depending on the liberation of soluble iodate from silver iodate added to the chloride-containing liquid. On addition of potassium iodide, six atoms of iodine are liberated for each chloride ion originally present. The determination of the iodine makes possible the accurate estimation of chloride in small amounts of blood or plasma.

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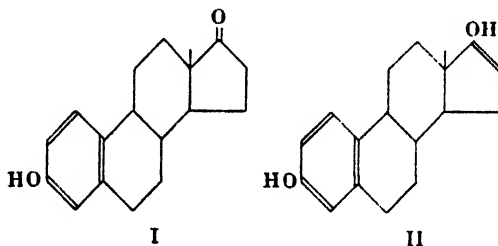
CXXXII. THE ABSORPTION SPECTRA OF OESTRONE AND RELATED COMPOUNDS IN ALKALINE SOLUTION.

By ROBERT KENNETH CALLOW.

From the National Institute for Medical Research, Hampstead, London.

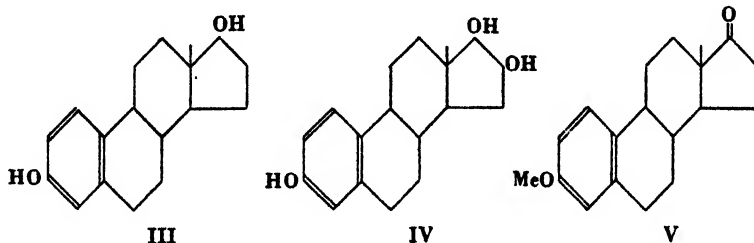
(Received April 1st, 1936.)

PEDERSON-BJERGAARD AND SCHOU [1935] recently measured the absorption spectrum of oestrone in aqueous-alcoholic solution and found that the peak of the absorption band moved from about 2800 Å. in acid or neutral solutions to about 2950 Å. in alkaline solution, whilst the intensity also increased slightly. They attributed this change to enolisation of the 17-keto-group and the influence of the new ethenoid linkage produced, as shown in formulae I and II.



It seemed improbable that introduction of an isolated ethenoid linkage would produce this change in absorption spectrum, which might better be attributed to salt formation by the phenolic 3-hydroxy-group. Strictly analogous changes occur in the case of simple phenols when they form phenoxides, as has been shown by Baly and Ewbank [1905] and by Ley [1920].

The matter was, therefore, put to the test by measuring the absorption spectra, in neutral and alkaline aqueous-alcoholic solution, of two oestrin derivatives in which tautomerism in ring IV is impossible, but which can form phenoxides: namely oestradiol (III) and oestriol (IV). Oestrone methyl ether (V) in which enolisation is conceivable but salt formation is impossible was also investigated. The measurement of the absorption spectrum of oestrone was repeated under the same conditions.



As may be seen in the diagrams (Fig. 1), oestrone, oestradiol and oestriol all behave similarly. One molecular proportion of NaOH in the concentration used

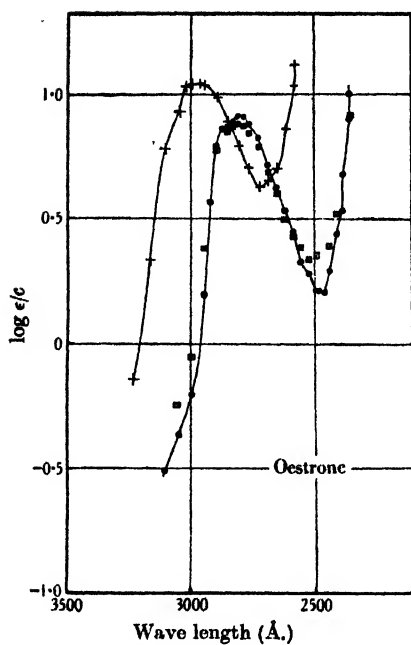


Fig. 1 a.

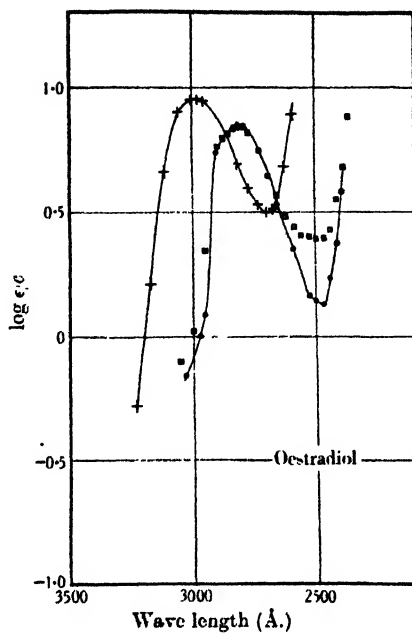


Fig. 1 b.

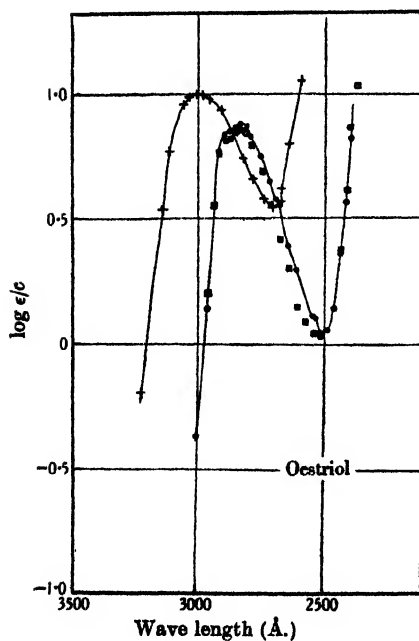


Fig. 1 c.

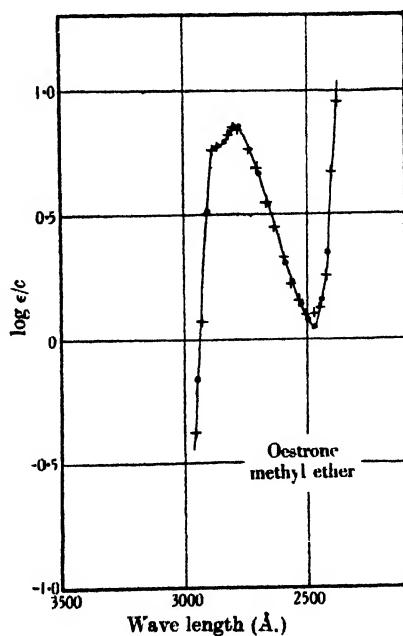


Fig. 1 d.

Fig. 1. Absorption spectra of oestrone (a), oestradiol (b), oestriol (c), and oestrone methyl ether (d) in 90% alcohol; neutral •, N/3000 NaOH ◻, N/100 NaOH +.

(*ca.* $N/3000$) produced little effect, whilst 30 molecular proportions shifted the maxima to 2950–3000 Å. (Fig. 1, *a, b, c*). On the other hand, the absorption spectrum of oestrone methyl ether was not affected by an excess of NaOH (Fig. 1 *d*). It is, therefore, demonstrated that the change in absorption spectrum in alkaline solution is to be attributed to the formation of phenoxide. No evidence is afforded whether enolisation occurs or not. It may be pointed out that this method of examination is diagnostic of a free phenolic hydroxy-group, and should be applicable to natural esters of oestrone or oestriol (such as the oestriolglucuronic acid of Cohen and Marrian [1936]) in the same way that absorption spectra have been applied to the determination of the constitution of the purine nucleosides by Gulland *et al.* [1934].

EXPERIMENTAL.

Materials. The M.P. (uncorr.) of the substances used were as follows: (*a*) oestrone, 255–257° ($[\alpha]_D^{25}$, +165°); (*b*) oestradiol, 170–172°; (*c*) oestriol, 279–280°; (*d*) oestrone methyl ether, 167–169°: specimens (*a*) and (*c*) were given by Dr A. Girard to Dr O. Rosenheim, to whom I am indebted for passing them on, (*b*) was purchased from British Drug Houses, Ltd., (*d*) was prepared from oestrone as described by Cohen *et al.* [1935].

Measurements. The absorption spectra were measured by the method of Philpot and Schuster [1933], and are expressed as $\log \frac{\epsilon}{c}$ where

$$\epsilon = \frac{1}{d} \log_{10} \left(\frac{\text{incident}}{\text{transmitted}} \text{ light} \right) - \epsilon_0$$

and *c* is the concentration in g. per l.

The substances were made up in about 0.01 % solution in absolute alcohol. 5 ml. portions of this were diluted with (i) 0.5 ml. of water, (ii) 0.5 ml. of aqueous $N/300$ NaOH and (iii) 0.5 ml. of aqueous $N/10$ NaOH and photographed. Comparison spectrographs of the solvent were made from the same mixtures of alcohol with aqueous NaOH. The actual final concentrations used were: oestrone, 0.00969 %; oestradiol, 0.00960 %; oestriol, 0.00949 %; oestrone methyl ether, 0.00936 %.

SUMMARY.

Addition of sodium hydroxide to solutions of oestrone, oestradiol or oestriol causes a shift of the ultraviolet absorption band towards the visible region. The absorption of oestrone methyl ether is unaffected. The change in absorption is therefore due to salt formation by the phenolic group in the 3-position, and is not to be explained in the case of oestrone by enolisation of the 17-keto-group.

I am indebted to Mr E. H. Pitte for technical assistance in these measurements.

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CXXXIII. STUDIES IN IMMUNO-CHEMISTRY.

I. THE PREPARATION AND PROPERTIES OF A SPECIFIC POLYSACCHARIDE FROM *B. DYSENTERIAE* (SHIGA).

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THE recognition of the important part played by polysaccharide substances in the specific reactions that are induced by bacterial antigens, such for example as those which take place between virulent forms of bacteria and the homologous antibacterial immune-sera, followed the observation of Dochez and Avery [1917] that culture filtrates from the three main types of *Pneumococcus* contain a soluble substance which gives specific precipitation with the homologous immune-serum. The serologically reactive material was subsequently isolated and identified by Heidelberger and Avery [1923; 1924; 1925] and Avery and Goebel [1933] as a complex polysaccharide which was contained in the bacterial capsule and which was specific for each of the types of *Pneumococcus* investigated. Furthermore, their work indicated that the specific polysaccharides are not antigenic, but that as they exist in the intact bacterial cell they are almost certainly bound with some other substance—most probably a protein—and that it is the resulting complex which possesses antigenic properties.

The conception of antigenic structure which attributes the specific immunological properties of a complete bacterial antigen to a polysaccharide constituent and the antigenicity of the bacterial complex to a combination of this constituent with some other substance is now supported by a considerable body of evidence which, when considered in conjunction with studies on bacterial variation, has gradually led to the idea that the essential difference between the antigen of a virulent and of an avirulent organism is the presence in the former of a specific polysaccharide which is not found in the avirulent strain. Substances, such as the specific bacterial polysaccharides, that do not possess complete antigenic power but have the capacity of reacting with immune-sera prepared against the whole antigen, have been termed haptens by Landsteiner.

As soon as it was generally recognised that the primary function of the polysaccharide portion of a bacterial antigen is to endow the antigen with its characteristic immunological specificity it became of considerable importance to ascertain the influence exerted by certain chemical groupings within the polysaccharide on the specificity of the whole antigenic complex.

Considerable advances have already been made in our knowledge of the influence of the structure and of the stereochemical configuration of simple carbohydrates on the specificity of artificial carbohydrate-protein antigens [Goebel and Avery, 1929; Goebel *et al.*, 1932; 1934], but it must be admitted that as yet we possess little definite insight into the essential factors that are responsible for the strict specificity, or for overlapping specificity where this exists, that is shown by the serologically reactive bacterial polysaccharides. Goebel [1935] attempted to find a chemical basis for the immunological cross-reactions

that are displayed by the specific polysaccharides of *Pneumococcus* Types III and VIII, and his investigations revealed that the polysaccharides of these strains contain an identical uronic acid component and that this molecule is most probably the common structure within each of the polysaccharides which gives rise to the overlapping specificity.

With the view of obtaining further knowledge concerning the nature of the carbohydrate groupings that are associated with immunological specificity in bacterial antigens a study has been made of the specific polysaccharide of *B. dysenteriae* (Shiga). In a previous paper [Morgan, 1931, 1] a preliminary account was given of the isolation of a specific polysaccharide from this organism. Since then a number of modifications in the technique of isolation and purification have been introduced, and these, together with the properties of the purified specific polysaccharide, are described in this communication.

An examination of the properties of the crude, protein-free polysaccharide preparations isolated from Shiga's bacillus by the original process showed that they were mixtures of several polysaccharides [Morgan, 1931, 2]. The introduction of certain modifications in the methods of isolation and purification of the specific polysaccharide has made it possible to separate at least three non-specific polysaccharides from their association with the specific substance. Two of these polysaccharides give specific precipitation with an anti-agar serum up to a very high dilution and, therefore, appear to be related to the agar on which the dysentery bacillus was grown. An examination of these substances has shown that they do not consist of unchanged agar but that they are most probably soluble agar derivatives which still retain the serologically reactive configuration of groups that are present in the undisrupted agar molecule. It seems probable that these soluble forms of agar have been produced by certain enzymic processes associated with the growth of the dysentery bacillus. Another non-specific polysaccharide substance, which was found to be nitrogen-free and strongly dextrorotatory, was subsequently identified as glycogen.

EXPERIMENTAL.

The isolation of the specific polysaccharide.

The variant of *B. dysenteriae* (Shiga) that has been used throughout this work corresponds to the "smooth" form originally described by Arkwright [1921]. The organism yields a stable suspension in ordinary broth culture and in 2.5% saline, and it produces typical smooth, glistening colonies when grown on nutrient agar.

The bacilli from 250 Roux bottles were washed off with distilled water saturated with chloroform in the proportion of about 1 l. to 100 bottles. The final bacterial suspension was adjusted to p_H 4.0 with acetic acid and was immediately passed through a Sharples centrifuge, the clear supernatant was rejected and the organisms were resuspended in about one-tenth of the original volume of distilled water and stored at 0° in the presence of chloroform. This procedure was repeated until the growth from 1000 Roux bottles had been collected. The rejection of the first supernatant fluid, which contained most of the soluble products of bacterial metabolism, together with a proportion of the specific substance, eliminated practically the whole of the non-specific polysaccharides that made up so large a part of the protein-free mixed polysaccharide preparations which were isolated by the original method. The washed and resuspended bacteria were adjusted to p_H 4.0 by the addition of acetic acid and were heated at 100° for about 45 min. The suspension was again passed through

the supercentrifuge or filtered through chain-cloth to remove the bacterial debris and the coagulated bacterial protein. The process of extraction was repeated until the whole of the specific polysaccharide in the bacterial mass had been recovered. The method of isolation of the specific polysaccharide from the combined acid extracts was essentially the same as that which was described in the earlier communication. Since that time, however, the precipitation of the specific substance as a basic lead complex has always been carried out at 0° and the addition of the dilute ammonia carefully controlled in order to avoid an excess of alkali. The basic lead complex was immediately filtered off, washed with ice-cold water, resuspended in water and saturated with carbon dioxide. If the original supernatant culture fluid has been completely removed from the bacilli before they are heated in the acetic acid solution, only a small precipitate is produced when mercuric acetate is added to the acidified polysaccharide solution resulting from the decomposition of the basic lead complex with carbon dioxide. The addition of mercuric acetate at this stage to some preparations produced no precipitate. The test must be repeated on a small sample of the solution after it has been concentrated by evaporation to about one-tenth of its original volume; only if the test is then negative can the mercury purification be safely omitted from the main preparation. The final material, which was found to be protein-free, consists of the specific bacterial polysaccharide hapten contaminated with small amounts of various non-specific polysaccharides the last traces of which were removed as follows. The crude polysaccharide material was dissolved in ten parts by weight of water and glacial acetic acid was slowly added until a concentration of 90 % acetic acid was reached. The precipitate which formed possessed a relatively low specific activity and was removed by centrifuging. Acetic acid was then added to the clear supernatant fluid until the whole of the specific polysaccharide was precipitated; under these conditions this occurs between 90 and 96 % concentration of acetic acid. The material which remained in solution and which possessed only slight serological activity was recovered by the addition of several volumes of alcohol or ether, which precipitated it as a white flocculent mass. The specific polysaccharide substance was further purified by solution in 10 parts of water and treatment with acetic acid as described above until a 10 % aqueous solution of the substance gave no precipitate on the addition of ten times its volume of glacial acid; precipitation should, however, be complete when a further 10 parts of acetic acid are added. The specific material was again dissolved in 10 parts of water and was fractionated by the addition of acetone which was slowly added with constant shaking. A sticky oily precipitate first formed and was separated after the solution had remained at 0° overnight. The further addition of acetone produced a flocculent precipitate which could be readily separated by centrifuging and a final fraction was obtained by pouring the supernatant fluid into several volumes of acetone. The fractional precipitation of the crude material with acetic acid and subsequently with acetone removes all the non-specific polysaccharide substances. A preparation of the specific polysaccharide is not considered homogeneous until the first and last fractions obtained from the use of both these methods have been found to possess identical chemical and serological properties.

The final product, after several reprecipitations from aqueous or pyridine solution by alcohol, had $[\alpha]_D + 98^\circ$ ($c = 1.0\%$) and repeated fractionation from aqueous or pyridine solution with acetic acid, acetone, alkaline, neutral or acid alcohol failed to yield a polysaccharide fraction which possessed a different specific rotation or serological activity. The specific polysaccharide preparations isolated from two different strains of Shiga's bacillus were found to be identical

in all the chemical and immunological properties examined. During the course of this work more than 10,000 Roux bottles of growth have been investigated by the method described and the results have shown that from 1000 Roux bottles 1-2 g. of pure specific polysaccharide are usually obtained. The specific polysaccharide has been found to represent not more than about 20 % of the total polysaccharide material present in the original unwashed bacterial suspension.

Chemical properties of the specific polysaccharide.

The specific polysaccharide, $[\alpha]_D + 98^\circ$, after repeated precipitation from aqueous solution by alcohol or acetone, is obtained as a white amorphous substance. It can be heated at 100° in air or *in vacuo* without becoming discoloured. Analysis of the substance after drying at 100° over phosphorus pentoxide *in vacuo*: C, 41.9; H, 6.35; N, 1.61 %. The polysaccharide yields 97 % of reducing substances (Hagedorn and Jensen) calculated as glucose after acid hydrolysis. A specimen of the polysaccharide (0.64 g.) required 7.1 ml. 0.01 *N* caustic soda for neutralisation; the acid equivalent is therefore about 9000. An aqueous 2 % solution of the polysaccharide forms no precipitate when it is treated with solutions of the following reagents: tannic acid, barium hydroxide, mercuric acetate, uranium acetate, copper sulphate and lead acetate. The orcinol and phloroglucinol tests for pentoses, the naphthoresorcinol test for glycuronic acid and the salicylsulphonic acid, trichloroacetic acid and picric acid tests for proteins were uniformly negative when tested on 20 mg. samples of the specific substance dissolved in 0.5 ml. of water. The polysaccharide gives no coloration with Selivanoff's reagent or with iodine solution. It is not precipitated when warmed with Fehling's solution. After prolonged electrodialysis, using cellophane membranes and a potential of 120 v., the polysaccharide which was recovered was indistinguishable, in all the chemical and immunological properties examined, from the untreated substance. The specific polysaccharide dialyses very slowly through a cellophane membrane; the rate of diffusion however is too small to be of any practical value. A 1 % solution of the polysaccharide in acetic acid, p_H 2.5, is serologically active after being heated for several hours at 100° . The specific substance is slightly soluble in a saturated solution of ammonium sulphate and this property has occasionally been utilised as an alternative method for the removal of non-specific polysaccharide impurities.

In an earlier paper it was shown that treatment of the crude polysaccharide preparation with nitrous acid, under conditions similar to those used in Van Slyke's technique for the determination of amino-groups in amino-acids, failed to liberate the nitrogen contained in the specific polysaccharide complex. A similar experiment has now been made with a specimen of the pure polysaccharide; although the reagents were allowed to react for 18 hours at 35° there was no liberation of nitrogen nor was there any demonstrable alteration in the serological properties of the specific substance. Hynd and Macfarlane [1926] found that the action of nitrous acid on certain nitrogenous sugar derivatives could be considerably accelerated by the addition of mineral acid to the reacting mixture. A preliminary experiment showed that the prolonged action of sulphuric acid of higher concentration than 0.1 *N* caused destruction of the specific serological properties owing to hydrolysis of the polysaccharide. The reaction mixture was, therefore, made 0.05 *N* with sulphuric acid and allowed to react at 35° for 18 hours. The results given in Table I show that the nitrous acid has no action on the specific substance.

These experiments indicate that the nitrogen contained in the polysaccharide is most probably not present in the form of free amino-groups. This conclusion is

Table I.

The result of the action of nitrous acid on the specific polysaccharide
in presence of 0.05 *N* H₂SO₄ at 35° for 18 hours.

Property	Before treatment	After treatment
Specific rotation,* $[\alpha]_D$	+95°	+93°
Specific serological activity	1 : 12 × 10 ⁶	1 : 12 × 10 ⁶
Nitrogen (%)	1.62	1.70
Minimum anaphylactic dose (mg.)	0.015	0.010
Reducing power (H. and J.) (%)	2.4	2.2

* Uncorrected for ash.

rendered more certain when the stability of the nitrogen atoms to the action of alkali is considered. It has been found that whereas glucosamine and chondrosamine—carbohydrate substances which contain free amino-groups—yield practically the whole of their nitrogen in the form of ammonia on heating for a few minutes with normal alkali at 100°, the specific polysaccharide behaves under these conditions like the *N*-substituted substances, acetylglucosamine and acetylchondrosamine, which evolve only a small proportion of their nitrogen. Further evidence for the existence in the specific polysaccharide of substituted amino-groups only will be given when the results of the acid hydrolysis experiments are considered.

The action of emulsin, malt-diaxase, takadiaxase and a commercial preparation of cellulases and hemicellulases called "Luizyme" on an aqueous solution of the specific polysaccharide was investigated. In no case was there any detectable decomposition of the substance as determined by an increase in reducing power or by the usual serological precipitation technique. The decomposition and inactivation of the specific polysaccharide by a species of *Myrococcus* has already been described [Morgan and Thaysen, 1933].

Acetylation of the polysaccharide.

The thoroughly dried and finely powdered polysaccharide (0.50 g.) was added to a mixture of freshly distilled pyridine (16 ml.) and "A.R." acetic anhydride (10 ml.). The material dissolved after 5 minutes' heating at 50° to yield a pale yellow solution; the heating was continued for 1 hour at 70°. The pyridine solution was then cooled and poured into 250 ml. of ether. The fine buff-coloured precipitate of the acetyl compound was filtered off, washed successively and thoroughly with water (200 ml.) and alcohol-ether and dried *in vacuo* over sulphuric acid. The aqueous washings failed to give specific precipitation when they were diluted 1 : 100 or 1 : 1000 with saline and were mixed with an equal volume of a 1 : 4 dilution of a *B. dysenteriae* (Shiga) serum; it seemed therefore that the whole of the specific polysaccharide had been converted into the insoluble acetyl compound. The acetylated substance was dissolved in acetone (8 ml.), filtered to remove traces of insoluble material and re-precipitated by pouring into a mixture of 100 ml. of absolute alcohol and 50 ml. of dry ether. The solution of the substance in acetone and its reprecipitation with alcohol-ether mixture were repeated several times. Fractional precipitation of the acetyl polysaccharide from acetone or chloroform solution by the addition of ether showed that the derivative was homogeneous. The acetyl polysaccharide was obtained as a white amorphous solid; it was insoluble in methyl and ethyl alcohol, ether and water, but was readily soluble in chloroform and acetone; yield 0.66 g.; $[\alpha]_D^{16} + 71^\circ$ (in acetone, *c* = 0.5 %). Analysis: C, 48.0; H, 5.4 %.

Regeneration of the polysaccharide. The acetylated polysaccharide (0.6 g.) was dissolved in 30 ml. of aqueous acetone, and a slight excess of *N* KOH was added and the mixture kept at 37° for 4 hours. A test for serological activity showed that after this time the acetyl derivative had been hydrolysed and that the specific polysaccharide had been liberated. The solution was dialysed against distilled water until it was free from acetone and salts, evaporated to a volume of 10 ml. and poured into several times its volume of alcohol containing a trace of potassium acetate. The precipitated polysaccharide was filtered off, washed with absolute alcohol and dried *in vacuo*. The regenerated substance was very similar in chemical and immunological properties to the original polysaccharide. It gave specific precipitation with the homologous immune-serum at a dilution of 1 in 10⁷ and induced fatal anaphylactic shock when it was given intravenously to guinea-pigs that had been passively sensitised with immune-serum. The polysaccharide had $[\alpha]_D^{18} + 95^\circ$ and was insoluble in methyl and ethyl alcohol, acetone and chloroform. Analysis: C, 42.1; H, 6.5; N, 1.6 %.

Benzoylation of the polysaccharide.

The benzoylation was carried out by the method of Schotten and Baumann. To an aqueous solution of the polysaccharide (0.45 g. in 20 ml. water) benzoyl chloride was slowly added with vigorous stirring; the solution was kept slightly alkaline during the reaction by the addition of dilute sodium hydroxide. The benzoylated polysaccharide separated from the solution as a heavy white precipitate. After the reaction was complete a 1 in 100 dilution of the clear fluid failed to give a positive Molisch test or to give specific precipitation with a *B. dysenteriae* (Shiga) immune-serum. The precipitate of the benzoyl compound was filtered off, washed successively with 0.01 *N* NaOH, water and alcohol and dried *in vacuo* over sulphuric acid. The white amorphous material was dissolved in 5 ml. of 95 % acetone, centrifuged to remove traces of insoluble residue and reprecipitated from the acetone solution by the addition of 200 ml. of a mixture of equal parts of alcohol and ether, $[\alpha]_D^{18} + 63^\circ$ (in acetone).

The regeneration of the polysaccharide. An aqueous acetone solution of the benzoyl polysaccharide was treated with alkali exactly as described above for the regeneration of the polysaccharide from the acetate. The properties of the regenerated polysaccharide were similar to those found for the original material.

The serological properties of the specific polysaccharide.

The specific polysaccharide gives a definite precipitate up to a dilution of 1 : 12,000,000 with the homologous immune rabbit or horse sera but it fails to form a precipitate when it is mixed with a normal rabbit or horse serum, with a horse serum that contains a high titre of agar precipitins or with an immune rabbit serum which had been produced by the intravenous injection of formalised "rough" *B. dysenteriae* (Shiga). Serial dilutions of a homologous immune rabbit serum which had been repeatedly adsorbed with a suspension of washed "rough" *B. dysenteriae* (Shiga) gave precipitation to the same end-point as the unadsorbed serum when they were treated with an optimum concentration (1 : 100,000) of the specific polysaccharide. From these observations it would appear that the specific polysaccharide is not associated with the antigenic complex that is present in the "rough" variant. The absence of the serologically active polysaccharide from the carbohydrate material that has been isolated from the "rough" variant of Shiga's bacillus gives support to this conclusion. In order to obtain evidence that the specific polysaccharide, in the form in which it

has been isolated, accounts for all the serological phenomena of specificity associated with the undisrupted antigenic complex of the "smooth" form of *B. dysenteriae* (Shiga), the following experiments were made. A homologous antibacterial rabbit serum (10 ml.), which had been repeatedly adsorbed with a dense washed suspension of "rough" *B. dysenteriae* (Shiga), was treated at 37° with a 1:5000 dilution of the specific polysaccharide until the serum no longer produced a precipitate on the subsequent addition of the polysaccharide solution. The serum was kept at 0° for 24 hours and filtered at this temperature through a Berkefeld filter candle. The addition of a further quantity of the specific substance produced no precipitate after the adsorbed serum had stood for 2 hours at 37° and overnight in the cold room. Two other immune-sera were treated in the same manner; untreated samples of the three immune-sera were used as controls.

A dense freshly prepared suspension of *B. dysenteriae* (Shiga) was divided into three (50 ml.) portions which were adjusted to p_H 4.5, 7.0 and 8.5 respectively by the addition of an equal volume of an appropriate phosphate buffer solution. The bacterial suspensions were again divided into equal (50 ml.) parts. Three of the portions, at p_H 4.5, 7.0 and 8.5 respectively, were heated at 100° for 10 min. and filtered at once through small sterile Seitz filters. The three remaining portions were allowed to autolyse at 37° in the presence of toluene for 72 hours and were then filtered in the same manner. A series of ten-fold dilutions in saline of each of the six filtrates was tested against the adsorbed and unadsorbed antibacterial sera. The results obtained with each set of three serum samples were identical: one series of results is given in Table II. It will be seen that

Table II.

The results obtained when extracts of *B. dysenteriae* (Shiga) are added to an antibacterial serum from which the precipitins for the specific polysaccharide and the "rough" antigenic complex had been removed by adsorption.

Treatment of <i>B. dysenteriae</i> (Shiga) sus- pension	Dilution of filtrate tested								
	1:10			1:100			1:1000		
	Unadsorbed serum			Adsorbed serum					
Heated 100° 10 min.	p_H								
	4.5	##	##	+	+	tr	tr	0	0
	7.0	##	+	+	0	0	0	0	0
Autolysed 35° 72 hours	8.5	##	+	+	tr	0	+	0	0
	4.5	##	+	+	0	0	0	0	0
	7.0	##	+	+	tr	0	tr	0	0
	8.5	##	##	+	+	tr	+	0	0
##, very heavy precipitation.						+, heavy precipitation.			
+, distinct precipitation						tr, trace of precipitation.			

in no instance was there any appreciable precipitation with the adsorbed serum, a result which indicates that simple extracts or autolysates of the bacterial cell made at an acid, neutral or alkaline reaction do not contain a substance which will precipitate with a serum which has been thoroughly adsorbed with the purified specific polysaccharide and the "rough" antigenic complex. Furthermore, the unadsorbed sera agglutinated suspensions of *B. dysenteriae* (Shiga) to a titre of about 1 in 4000 but after precipitation with the purified specific polysaccharide complete agglutination failed to occur at a higher dilution than 1:10. It appears therefore that treatment of an antibacterial *B. dysenteriae* (Shiga) serum with the specific polysaccharide until the whole of the specific

precipitins for this polysaccharide hapten have been removed also completely removes the agglutinins for the intact homologous organisms.

The specific polysaccharide has been found to possess the property of neutralising the haemolytic action of 'Shiga' heterophile antibody on sheep red-cells as well as of combining with the homologous antibacterial immune-substance. The property of neutralising these two distinct types of antibody suggests that the 'Shiga' polysaccharide contains in each of its molecules two kinds of determinant receptors of diverse specificity [Meyer and Morgan, 1935].

Acid hydrolysis of the specific polysaccharide.

The specific polysaccharide is slowly hydrolysed by dilute mineral acid with the liberation of 97 % by weight of reducing substances calculated as glucose and with the complete loss of its specific immunological properties. Fig. 1 shows the results of a hydrolysis experiment with 0.1 *N* sulphuric acid at 100°. The progress

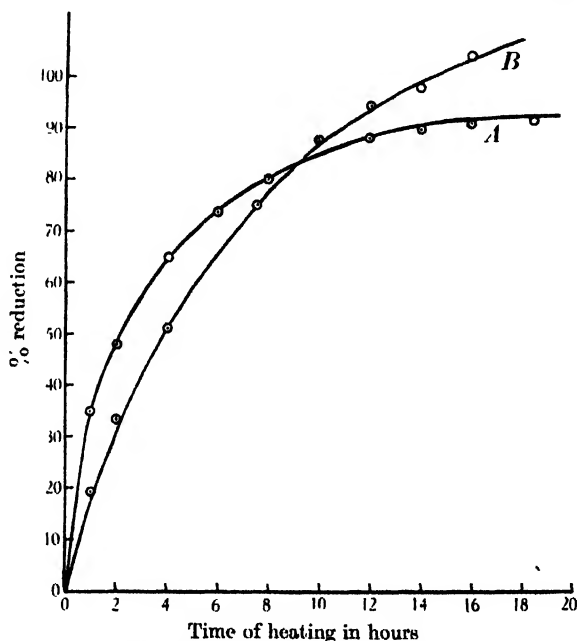


Fig. 1. A, reducing power (Hagedorn and Jensen); B, reducing power (hypoiodite method).

of the hydrolysis was followed by estimating the reducing power of the solution by the Hagedorn and Jensen method and by the sodium hypoiodite method as modified by Macleod and Robison [1929]. The reducing power determined by the latter method is, in the absence of certain groups within the molecule, a measure of the free aldehyde groups liberated during the course of the hydrolysis. It will be seen from the curves that during the early stages of the hydrolysis the H. and J. reducing power was almost twice as great as the reducing power of the solution towards the alkaline solution of iodine. The iodine reduction, however, rises more rapidly towards the end of the hydrolysis than the H. and J. reduction and, after about 10 hours' heating, is equal in amount to the reducing power as determined by the latter method. The simplest explanation of the results is that early during the hydrolysis the polysaccharide complex is easily and rapidly broken down

into smaller structural units. The existence of reducing oligosaccharides in the solution during the preliminary stages of the hydrolysis would give rise to a set of reducing values similar to those recorded in Fig. 1 for the hydrolysis products of the polysaccharide. The further action of the acid finally results in the complete breakdown of the intermediate structures into simple hexoses.

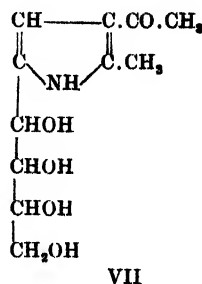
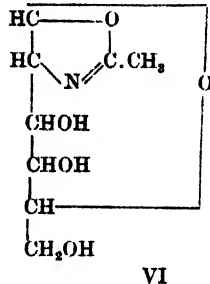
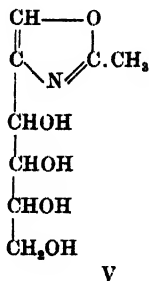
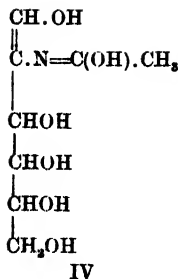
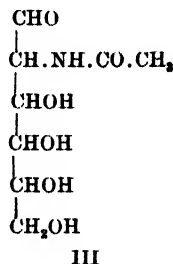
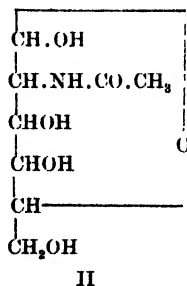
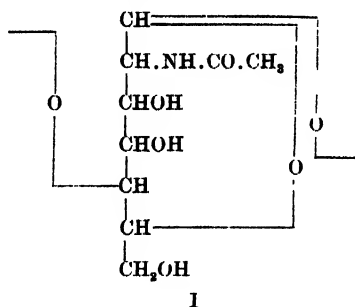
From the analytical figures obtained for the purified polysaccharide and from other evidence already mentioned it would appear that the whole of the nitrogen present in the complex is in the form of substituted amino-groups and that most probably one of these groups is associated with one out of every five hexose units which constitute the polysaccharide structure.

The following experiment was made in order to identify the nature of the substituent group attached to the amino-group in the specific polysaccharide. A specimen of the polysaccharide (0.9 g.) which had been repeatedly reprecipitated, first from weakly alkaline alcohol and then from neutral alcohol, was dissolved in 10 % sulphuric acid (25 ml.) heated at 100° for an hour and distilled in a current of steam. The distillate was allowed to run into an aqueous suspension of freshly prepared silver carbonate which was contained in a closed receiver kept at 0°. When the distillation was complete the suspension was warmed slightly, the excess of silver carbonate was removed, the filtrate was evaporated *in vacuo* to 5 ml. and 15 ml. of alcohol were added. After standing at 0° for 18 hours a mass of colourless needles separated; they were filtered off and again crystallised from 75 % alcohol. Analysis (material dried *in vacuo*). (Found: C, 14.5; H, 2.3; Ag, 64.2 %; silver acetate requires C, 14.4; H, 1.8; Ag, 64.7 %). The crystals appeared to be identical with an authentic specimen of silver acetate which had been crystallised under similar conditions. From this experiment the *N*-substituted hexosamine present in the polysaccharide is identified as a *N*-acetylhexosamine. The quantitative estimation of acetyl groups was made according to the method of Pregl and Soltys [1929]. (Found: 4.92 and 5.09 % CO.CH₃. Calculated for a polysaccharide composed of units each of which consist of four hexoses combined with a molecule of acetylaminohexose, CO.CH₃, 5.03 %). Furthermore the results indicate that the whole of the nitrogen contained in the polysaccharide (1.61 %) is present in the form of acetylated amino-groups.

During the acid hydrolysis of the polysaccharide with 0.1 *N* sulphuric acid there is a gradual elimination of the acetyl groups from the *N*-acetylhexosamine molecules with the consequent formation of primary amino-groups which react with iodine in alkaline solution and give rise to false reducing figures. This is clearly seen from the iodine reduction curve shown in Fig. 1 where the reduction calculated as glucose rises towards the end of the hydrolysis to a value well over 100 %.

In an attempt to follow the rate of liberation of *N*-acetylhexosamine during the acid hydrolysis of the polysaccharide, Morgan and Elson [1934] developed a method whereby this compound could be quantitatively determined in the presence of the mixed hydrolysis products. It was soon found, however, that the *N*-acetylaminohexose which was liberated during hydrolysis with 0.1 *N* sulphuric acid was readily converted by the further action of the acid into the corresponding deacetylated aminohexose. In order to follow this additional hydrolytic change it became necessary to devise a quantitative procedure for the determination of aminohexoses. The work of Pauly and Ludwig [1922] had already shown that glucosamine could be converted into a pyrrole derivative by heating with an alkaline solution of acetylacetone so that it was only necessary to determine the optimum conditions for the quantitative conversion of glucos-

amine or chondrosamine into their corresponding pyrrole derivatives in order to elaborate a method for the determination of these two aminohexoses or presumably any other simple 2-aminohexose. The method, which has already been described in detail [Elson and Morgan, 1933], has enabled the whole of the nitrogen present in the final hydrolysis products of the polysaccharide to be identified as aminohexose. By the use of these methods for the determination of *N*-acetylaminohexose and aminohexose at different stages of the hydrolysis it has been found that during the early stages of the acid hydrolysis of the specific polysaccharide the products, after heating for a few minutes in alkaline solution, develop a reddish purple coloration following the addition of hydrochloric acid-*p*-dimethylaminobenzaldehyde in acetic acid solution. The unhydrolysed and the completely hydrolysed polysaccharide fail to develop the coloration under the same conditions. Furthermore, the treatment of the final hydrolysis products with an alkaline solution of acetylacetone causes a bright red colour to appear when an alcoholic hydrochloric acid solution of *p*-dimethylaminobenzaldehyde is added. The unhydrolysed polysaccharide does not give the coloration when it is treated under similar conditions. The above experimental observations can be readily explained as being due to the presence of *N*-acetylhexosamine units (Formula 1) in the undisrupted polysaccharide complex. The inability of the unhydrolysed polysaccharide to develop the reddish purple colour is due to the absence of the terminal aldehyde group from the *N*-acetylhexosamine units owing to the involvement of these groups in the glycosidic linkages which join the acetylhexosamine molecules to their neighbouring hexoses. It has been found that 1-methyl-*N*-acetylglucosamine, which likewise does not possess a free reducing group, also fails to give rise to the red-coloured complex. During the early stages of the hydrolysis, however, the polysaccharide breaks down into a number of smaller structural units among which single hexoses may exist. In any event, the *N*-acetylhexosamine molecule (II) is now found to possess a reducing aldehyde group as shown in structure (III), which, in the presence of



alkali, presumably changes to the enolic structure (IV) and eliminates water with the formation of a heterocyclic ring structure (V) or (VI), and it is the 2:4-disubstituted oxazole structure (V) or oxazoline structure (VI) which condenses with the *p*-dimethylaminobenzaldehyde and gives rise to the reddish-purple coloration (unpublished observations).

When, however, hydrolysis of the polysaccharide is complete, the acetyl groups are found to be completely eliminated from the *N*-acetylhexosamine molecules with the result that the formation of a heterocyclic ring structure similar to that suggested in the above scheme cannot take place and therefore no coloration develops with the *p*-dimethylaminobenzaldehyde reagent. If the resulting deacetylated hexosamine is now heated with an alkaline solution of acetylacetone, condensation is able to take place with the formation of 2-acetyl-3-methyl-5- α : β : γ : δ -tetrahydroxybutylpyrrole (VII) which readily condenses with *p*-dimethylaminobenzaldehyde to yield the red-coloured complex. It will be seen that with the unhydrolysed polysaccharide the formation of a substituted pyrrole derivative similar to (VII) cannot occur owing to the absence of a primary amino-group in the molecule. The presence of a reducing group in position 1 of the aminohexose does not appear to be necessary for the condensation to take place since it has been observed that 1-aminoglucose readily condenses with acetylacetone to form a pyrrole derivative.

The destruction of the specific immunological properties of the polysaccharide during hydrolysis.

Table III shows that after heating a 1 % solution of the polysaccharide with 0.1 *N* sulphuric acid for 60 min. at 100°, the serological property of forming a specific precipitate when it is mixed with an antibacterial 'Shiga' rabbit serum

Table III.

Showing the destruction of the serological activity of the specific polysaccharide during the course of hydrolysis with 0.1 *N* sulphuric acid at 100°.

Time min.	Dilution of polysaccharide giving specific precipitation with antibacterial rabbit serum									
	2×10^3	2×10^4	1×10^5	2×10^5	1×10^6	2×10^6	6×10^6	12×10^6	15×10^6	
0	---	+	##	##	+	+	+	+	±	
15	---	+	+	+	+	+	tr	0	0	
30	---	+	+	+	tr	0	0	0	0	
45	---	+	tr	0	0	0	---	---	---	
60	tr	tr	0	0	0	---	---	---	---	
90	tr	?tr	0	0	---	---	---	---	---	

Signs as in Table II.

has been completely destroyed. From the acid hydrolysis curve, Fig. 1, it will be seen that after 60 minutes' heating, the reducing power of the solution had risen to about 36 % as measured by the H. and J. method, and 18 % as measured by the hypiodite method: both these figures are calculated on the weight of the polysaccharide. These results indicate that as soon as the polysaccharide complex is split into smaller molecules, the specific serological activity, as measured by the precipitation test, disappears. It has been found, however, that the acid hydrolysis products of the polysaccharide at this stage of the hydrolysis cause a specific inhibition of precipitation when they are added to an antibacterial "Shiga" rabbit-serum before the addition of the intact specific polysaccharide.

Furthermore the hydrolysis products, when injected intravenously into guinea-pigs which had been passively sensitised with an antibacterial *B. dysenteriae* (Shiga) serum, caused the animals to become desensitised so that a subsequent injection of unchanged polysaccharide which would normally bring about a rapid and fatal anaphylactic shock, failed to induce anaphylaxis.

The inhibition experiments were carried out as follows. The partial hydrolysis products were neutralised with dilute alkali and diluted with physiological saline to yield a 0.1 % solution. An equal volume (0.25 ml.) of immune-serum was added to several different dilutions of this stock 0.1 % solution and the mixtures were incubated at 37° for 2 hours. To each mixture 0.25 ml. of a 1:10⁵ dilution of the specific polysaccharide was then added and the tubes were again kept at 37° for 2 hours. After standing at 0° overnight the tubes were examined for the presence or absence of a specific precipitate. In order to determine more closely the point at which the power of the polysaccharide to form a precipitate with immune-serum is completely destroyed, a number of hydrolysis experiments were carried out using different acid concentrations. When 0.01 *N* hydrochloric acid was used the polysaccharide after heating for 16 hours at 100° failed to precipitate with immune-serum but inhibited precipitation and anaphylaxis more efficiently than after it had been heated with 0.1 *N* sulphuric acid at 100° for 1 hour.

Attempts have been made, by means of simple dialysis experiments, to separate the active constituent of the hydrolysis product which is able to cause the specific inhibition of the combination of the polysaccharide with its homologous antibody. The results of inhibition tests carried out with the dialysate and with the bag contents of a typical dialysis experiment through cellophane are shown in Table IV. It is evident that both the non-diffusible bag contents and

Table IV.

Inhibition of the homologous antigen-antibody reaction by the partial hydrolysis products of the specific polysaccharide.

Material	Concentration mg. per 1 ml.	Final dilution of the acid hydrolysis products						
		1: 750	1: 1500	1: 2250	1: 3000	1: 3750	1: 4500	1: 5250
		Degree of inhibition						
Bag contents	3.3	c	c	c	i	tr	o	o
Dialysate	4.2	c	c	c	ac	i	o	o

c, complete inhibition.

ac, almost complete inhibition.

i, incomplete inhibition.

tr, trace of inhibition.

o, no inhibition.

the dialysate completely inhibit the formation of a precipitate even when the unchanged polysaccharide is added in optimum concentration (1:10⁵). Dilutions of the hydrolysis products greater than 1:3000 cause only a partial inhibition of precipitation.

By the use of 0.01 *N* hydrochloric acid it is possible to avoid the elimination of the acetyl group from the *N*-acetylhexosamine during the hydrolysis and, at the same time, to cause the destruction of the whole of the glycoside linkages that exist between the potential aldehyde groups of the *N*-acetylhexosamine units and the neighbouring hexoses. After 16 hours' heating practically the whole of the *N*-acetylhexosamine in the polysaccharide can be estimated by the colorimetric method of Morgan and Elson, a result which indicates that the acetylhexosamine has been liberated in the form of single molecules or, as is more probable, as reducing end-units in the oligosaccharides which have been formed.

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A more detailed account of the acetylhexosamine and hexoses that are liberated during the acid hydrolysis of the polysaccharide will be given in a later communication.

The presence of agar-like substances in the crude specific polysaccharide preparations.

During some experiments which were made in 1931 and in which the specific inhibition caused by the partial hydrolysis products of the specific polysaccharide material was first demonstrated, it was noticed that the hydrolysis products continued to yield a precipitate when they were treated with a *B. dysenteriae* (Shiga) horse serum although no precipitate was formed when the corresponding rabbit serum was employed. The precipitation tests that were set up with immune rabbit serum showed that the specific serological properties of the polysaccharide were rapidly destroyed whereas the precipitation tests carried out with the immune horse serum indicated that after a rapid fall in activity during the first 30 min. the precipitating activity of the specific polysaccharide appeared to remain constant at a level of about 1 : 500,000. The results of the precipitation tests are shown in Tables V and VI.

Table V.

Precipitation tests using an antibacterial *B. dysenteriae* (Shiga) rabbit serum.

Time of heating hours	Final dilution of partially hydrolysed crude bacterial polysaccharide preparation (0.25 ml.)						
	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6	3×10^6	6×10^6
0	+	+	+++	++	+	+	+
0.25	+	+	+++	+	+	+	0
0.50	+	+	+	+	+	0	0
0.75	+	+	+	0	0	0	0
1.0	+	tr	0	0	0	0	0
1.5	?	0	0	0	0	0	0

Signs as in Table II.

Table VI.

Precipitation tests using antibacterial *B. dysenteriae* (Shiga) horse serum (commercial sample).

Time of heating hours	Final dilution of partially hydrolysed crude bacterial polysaccharide preparation (0.25 ml.)						
	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6	3×10^6	6×10^6
0	++	++	++	+	+	+	+
0.25	++	++	++	+	+	tr	0
0.50	++	++	++	+	+	0	0
0.75	++	++	+	+	tr	0	0
1.0	++	+	+	+	tr	0	0
1.5	++	+	+	+	tr	0	0
2.0	++	+	+	+	0	0	0

Signs as in Table II.

At that time it was not possible to explain these experimental results, but the observations of Sordelli and Mayer [1931], who found that agar adsorbed on bacteria is rendered antigenic and produces agar antibodies in animals that are immunised with these organisms, suggested that the antibacterial horse serum that had been employed by us as a precipitating serum contained a high titre of agar precipitins.

An examination of several samples of anti-dysentery horse serum showed that they possess a considerable titre of anti-agar bodies and yield precipitates with high dilutions (1 : 500,000) of agar. Anti-dysentery serum is frequently produced on the large scale for therapeutic use by the intravenous inoculation of horses with killed cultures of *B. dysenteriae* (Shiga) which have grown on nutrient agar. During the immunisation agar precipitins are slowly formed and after several courses of injections, which would normally extend over several months, a potent anti-agar serum is produced. The absence of agar antibodies from the immune rabbit serum is presumably due to the short course of immunisation which they receive as compared with the intensive and repeated series of inoculations usually given to horses that are producing therapeutic sera for general use. It has been observed that for the production of a precipitating anti-meningococcus serum in rabbits it is necessary to administer a very prolonged course of immunisation and under these circumstances the resulting rabbit serum has occasionally been found to contain agar-precipitins.

The anti-agar horse serum that has been used in the experiments described in this paper was obtained from a horse that had been immunised with agar-grown meningococci and was therefore free from precipitins which would react with the specific polysaccharide of *B. dysenteriae* (Shiga).

It follows from these serological observations that the specific polysaccharide preparations in use at that time were contaminated with agar-like polysaccharide substances and therefore reacted with the anti-agar bodies present in the immune horse serum. In our experience specific polysaccharide preparations which have been obtained from organisms that have grown on agar always react with the formation of a precipitate when they are mixed with an anti-agar immune serum unless a very thorough chemical purification has been carried out.

THE NON-SPECIFIC POLYSACCHARIDES.

The isolation and identification of the various non-specific polysaccharide substances present in the supernatant fluid obtained from the original bacterial suspension has been considered to be of subsidiary importance in this investigation and for this reason they have only been superficially examined and will only be briefly discussed.

The first supernatant fluid, after the bacilli had been removed, was treated with normal and basic lead acetate and with mercuric acetate in the same manner as has been described for the isolation of the specific polysaccharide. From the supernatant fluid obtained from one batch of bacterial growth (1000 Roux bottles) about 10 g. of mixed, protein-free, non-specific polysaccharide was obtained. Fractional precipitation of the mixture with acetic acid yielded several fractions, the $[\alpha]_D$ of which varied from -60 to $+20^\circ$. The further fractional precipitation of an aqueous solution of each of these fractions, first with acetic acid and finally with acetone has yielded three apparently homogeneous polysaccharide substances; their properties are shown in Table VII.

Table VII. *The properties of the non-specific polysaccharides.*

Polysaccharide	$[\alpha]_D$	Nitrogen %	Greatest dilution of fraction giving precipitate with	
			<i>B. dysenteriae</i> (Shiga) serum	Anti-agar serum
1	-60°	5.2	0	1 : 5,000,000
2	$+20$	0.6	0	1 : 10,000,000
3	$+195$	0.0	0	0

Polysaccharide fraction no. 3 has been identified as glycogen. Since the polysaccharides 1 and 2 were not exhaustively fractionated it is not possible to claim that the specific rotations are those of pure substances. A strongly laevorotatory substance, $[\alpha]_D - 78^\circ$, which is readily soluble in 96 % acetic acid and which possesses a very high nitrogen content (14.1 %) has also been isolated.

A detailed account of the agar-like polysaccharides that are associated with the specific polysaccharide of *B. dysenteriae* (Shiga) will be published later.

DISCUSSION.

The serological behaviour of the purified specific polysaccharide isolated from *B. dysenteriae* (Shiga) supports the view that this substance is responsible for the most important specific immunological properties of Shiga's bacillus and that it represents a further example of the class of bacterial substances, termed by Landsteiner haptens, which confer upon certain bacterial antigens their strict immunological specificity.

The addition of the purified polysaccharide to the homologous antibacterial serum removes by precipitation the whole of the precipitins engendered by the specific antigen associated with the smooth form of *B. dysenteriae* (Shiga). Furthermore, antibacterial sera that have been completely absorbed with the polysaccharide no longer give specific agglutination with suspensions of Shiga's bacillus nor do they yield precipitates when treated with simple acid, neutral or alkaline extracts or autolysates of the intact organism. These experimental results, when considered together, give strong support for the belief that the highly purified polysaccharide hapten accounts completely for the serological phenomena of specificity of *B. dysenteriae* (Shiga).

The specific substance of *B. dysenteriae* (Shiga) appears, therefore, to belong to a group of substances, the complex polysaccharides, which possess colloidal properties and high molecular weights and for which adequate criteria of homogeneity are difficult to prescribe and determine. It is impossible, therefore, to claim with certainty that the specific substance, in its present state of purity, is a single chemical compound. However, after each stage in its purification the polysaccharide preparation has shown a corresponding change in its chemical and physical properties together with an increase in serological activity as measured by specific precipitation with the homologous immune rabbit or horse serum and a diminution in the minimum amount of the polysaccharide substance necessary for the induction of a specific and fatal anaphylactic shock in guinea-pigs that have been sensitised with the homologous antibacterial serum from the rabbit [Morgan, 1932]. The purified specific polysaccharide, $[\alpha]_D + 98^\circ$, which has been described in this paper, has remained unchanged in properties following the application of several methods of purification. Thus, conversion of the specific polysaccharide into the acetyl or benzoyl derivative and its regeneration by treatment with alkali yields the specific substance unchanged in all the chemical and immunological properties examined. Furthermore, the prolonged action of nitrous acid in the presence of dilute mineral acid fails to liberate the nitrogen contained in the specific complex or to alter the immunological properties of the polysaccharide. Finally, the repeated fractionation of the specific substance from acetic acid solution or from aqueous or pyridine solution by alkaline, neutral or acid alcohol or acetone has also failed to yield a specific polysaccharide substance with different chemical and immunological properties. These facts when considered together with the other data presented suggest that the polysaccharide substance which possesses an optical rotation $[\alpha]_D + 98^\circ$ and a nitrogen content

of 1.61 %, is the specific bacterial polysaccharide haptén virtually freed from other non-specific polysaccharide impurities.

The properties of the specific polysaccharide, so far as they are known at present, support the belief that the basic structural unit in the polysaccharide aggregate consists of four hexose molecules and one *N*-acetylaminohexose molecule. The unhydrolysed polysaccharide gives an iodine reduction which is equivalent to 3.4 % of glucose and this reducing figure would be given by a polysaccharide which has one hexose end-unit, possessing a free aldehyde group, in each group of 30 of the hexoses that constitute the whole polysaccharide molecule. It would appear therefore that the basic hexose unit repeats itself six times in the polysaccharide molecule. The molecular weight of the polysaccharide calculated from the hypoiodite titration is about 5100.

The polysaccharide (10 mg.) gives only a very faint test—representing less than 0.05 mg.—for the presence of *N*-acetylaminohexose, whereas if the terminal reducing group of the polysaccharide were associated with a *N*-acetylaminohexose molecule a quantitative determination would show the presence of about 3.5 % of *N*-acetylhexosamine. The reducing end-unit in the polysaccharide structure is therefore not a *N*-acetylaminohexose molecule. Acid hydrolysis of the polysaccharide under conditions that do not remove the acetyl groups from the *N*-acetylhexosamine molecules, rapidly breaks the glycosidic linkages which join the aldehyde groups of the *N*-acetylaminohexose molecules with their adjacent hexoses. It has been found that when, under these conditions of hydrolysis, the reducing power reaches about 45 % (H. and J.) and 30 % (hypoiodite), the whole of the nitrogen in the polysaccharide can be estimated colorimetrically as *N*-acetylaminohexose, a result which indicates that each *N*-acetylhexosamine molecule now possesses a reducing aldehyde group.

When the specific substance is heated with dilute mineral acid the serological properties are rapidly destroyed and their destruction is complete when the reducing power of the solution has reached only about one-third of its final value. It would appear therefore that as soon as certain linkages within the polysaccharide molecule are broken and long before the whole of the component hexoses are liberated as single molecules the substance loses its power to precipitate with the homologous immune serum and to induce anaphylaxis in guinea-pigs that have been passively sensitised with an antibacterial *B. dysenteriae* (Shiga) rabbit serum.

The products of partial hydrolysis at this stage of the hydrolysis, however, still possess some degree of serological activity since when they are added in a dilution as high as 1 : 3000 to the immune serum they cause a specific inhibition of the precipitation which normally takes place on the subsequent addition of the appropriate quantity of the specific polysaccharide. Furthermore the partial hydrolysis products are also able to desensitise guinea-pigs which have been passively sensitised with immune serum and thus protect them against the anaphylactic shock that is induced by a subsequent intravenous injection of the intact polysaccharide. These experimental results suggest that the chemical structure which is responsible for the specificity of the polysaccharide remains intact after the whole of the *N*-acetylaminohexose in the polysaccharide has been liberated in the form of single molecules or, as appears more probable, as end-units in the oligosaccharides that are formed. Experiments designed to isolate from the hydrolysis products the chemical substance which has the power to inhibit the specific polysaccharide-antibody reaction are in progress.

During the course of this work several non-specific polysaccharide substances have been isolated from their association with the specific polysaccharide of

B. dysenteriae (Shiga). At least two of the non-specific polysaccharides react with immune sera which contain anti-agar bodies and these appear to be associated in some way with the agar upon which the organisms have been grown. The chemical and physical properties of these two serologically reactive but non-specific substances differ widely from the corresponding properties of agar, which indicates that they have been derived from the agar by enzymic processes which are most probably associated with the growth of the dysentery bacillus.

An examination of the literature reveals the frequency with which workers are content to designate their specific bacterial polysaccharide preparations "pure" provided that they are protein-free and essentially polysaccharide in nature, while the presence of non-specific polysaccharides and serologically reactive agar-like substances is frequently overlooked. It is suggested that serological tests for the presence of agar-like carbohydrate should be carried out on all specific polysaccharides that have been obtained from organisms grown on nutrient agar. Similarly, preparations of specific polysaccharide which have been derived from organisms that have been grown on solid (agar) medium or in broth cultures should be carefully examined for freedom from non-specific polysaccharides before they are accepted as "pure" for immuno-chemical studies.

SUMMARY.

1. A modified method is described for the isolation and purification of a specific polysaccharide from the "smooth" form of *B. dysenteriae* (Shiga).

2. The polysaccharide is strongly dextrorotatory, $[\alpha]_D + 98^\circ$, it contains 1.6% nitrogen the whole of which is present in the form of acetylated amino-groups, and it yields 97% of reducing sugars on acid hydrolysis.

3. The specific substance accounts completely for the serological specificity of *B. dysenteriae* (Shiga).

4. The occurrence and significance of certain non-specific polysaccharides is discussed.

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CXXXIV. VARIABILITY IN THE ACTIVITY OF BACTERIAL ENZYMES.

I. THE EFFECT OF THE AGE OF THE CULTURE.

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MANY papers have appeared during the last few years dealing with the dehydrogenase reactions induced by suspensions of washed bacterial cells. Up to the present no attempt has been made to examine systematically the activities of these enzymes in relation to any variations that might result from growing the organism under different conditions. In the past it has been more or less assumed that when grown on one of the usual bacteriological nutrient media an organism will exhibit a characteristic range of enzymic behaviour, although instances have been cited in which differences in this behaviour appeared to develop. Thus Klotz [1906] showed that a non-lactose-fermenting organism, *B. perturbans*, if grown upon a solid medium containing lactose, produced colonies which developed lactose-fermenting papillae. When the organism was sub-cultured from the central non-lactose-fermenting colony the resulting cells did not ferment this sugar, whilst subcultures from the lactose-fermenting papillae again formed colonies with a non-lactose-fermenting centre but with lactose-fermenting papillae. It would seem that under the conditions of these experiments an organism which was apparently unable to ferment lactose had, during growth upon a medium containing this sugar, developed the necessary enzymes to effect this change. Karström [1930] extended this work and showed that of the enzymes of bacteria that attack sugars some, the so-called "constitutive" enzymes, form an invariable part of the cellular biochemical structure, whilst others, the "adaptive" enzymes, are absent from the cells which have not been grown in the presence of their substrate. Stephenson and Stickland [1932] demonstrated that a suspension of washed cells of *Bact. coli* grown in broth containing added formate possess a hydrogenlyase which will decompose formate with the evolution of hydrogen, whilst the cells obtained from the same organism grown on plain broth do not possess this enzyme. Yudkin [1932] developed this work further. These results indicate the possible importance to its enzymic make-up of the constitution of a medium upon which an organism grows. In the present series of papers it is hoped to extend the enquiry into an analysis of various factors that might influence enzyme formation. The work described in this particular paper serves to show that the activities of dehydrogenases, when tested by the usual procedure, are not constant throughout the life of a culture, but appear to vary considerably with the time the organism is allowed to grow before it is reaped for enzyme examination.

EXPERIMENTAL.

The organisms examined in this paper were grown upon the usual nutrient media, *viz.* heart broth, caseinogen digest broth or nutrient agar. With the liquid medium good aerobic conditions were secured by placing 120 ml. in Roux bottles

and incubating them on their sides. For a particular series of experiments all flasks were inoculated, as far as possible, at the same time; if this was not possible the stock inoculum was stored at 0° for the short time (a few hours at most) during which it was being used. Every flask of medium received equal amounts of the stock inoculum, which was sometimes a broth culture and sometimes a suspension of the organism in quarter strength Ringer solution. The inoculated flasks were incubated at 37°, and a sufficient number were removed at the required intervals to ensure an adequate supply of washed organisms for the enzymic examinations. The cell suspensions were obtained in the usual way by centrifuging, or washing the cellular deposit twice with saline, suspending finally in quarter strength Ringer solution, aerating for 20 min. and examining within a short time for dehydrogenase activity. The number of inoculated flasks required to give sufficient organisms after a short period of growth was naturally much larger than the number required for longer periods of growth.

The Thunberg tubes contained 1 ml. of bacterial suspension, 1 ml. 1/10,000 methylene blue solution, 0.5 ml. substrate solution at p_H 7.4 and 0.5 ml. of phosphate buffer, p_H 7.4. The tubes were evacuated at the water-pump, immediately incubated at 45° and the time required for decoloration of 90% of the methylene blue noted. The end-point was obtained by matching a similar mixture of organism and nitrate containing only 1/10 as much methylene blue. All the bacterial suspensions of a given series were standardised by turbidity comparisons with standard suspensions of *Bact. coli* cells and were usually adjusted to an opacity of 5×10^8 cells per ml. of suspension. With *Bact. pullorum* and *Bact. suispestifer* thicker suspensions were required. All bacterial suspensions were examined within an hour or two of their preparation.

Variable dehydrogenase activity of Bact. coli grown on heart broth.

When suspensions of cells of *Bact. coli* grown on heart broth are prepared and examined in the way previously described it is found that their dehydrogenase activities are not constant but may vary considerably with the age of the culture from which the organism was obtained. This variability is not the same for all the enzymes as an examination of the ratio of the reduction times for any two donators throughout the series will show. Nevertheless, there seems to be a general tendency for the dehydrogenase activities to increase at first, reach a maximum and subsequently to decrease. These observations are illustrated by the results recorded in Table I. It will be seen that whilst the activity of the formic dehydrogenase remains practically constant throughout the course of the experiment, the activities of all the other enzymes examined vary to a greater or less degree. The reduction times obtained with the 4, 24, 48 and 72-hour organisms are respectively 8.9, 2.9, 2.5 and 3.5 min. for lactate, and 5.2, 3, 23 min. and > 2 hours for glucose; results which clearly illustrate both the variability of the activity of individual enzymes and the fact that this variability is not the same with each enzyme. This difference in variability is further emphasised by the ratios given in Table I, comparing one series of reduction times with another. With the amino-acid enzymes the peak in the activity is very marked. Of those examined the most active suspension, considering the enzymes collectively, appears to be that obtained from the organism grown for 24 hours. A comparison of the various reduction times obtained with this and with other suspensions (*vide* last column, Table I), emphasises the differences that may be obtained. In fact the differences may be so great that it would be a

Table I. *Dehydrogenase activities of suspensions of Bact. coli cells obtained from cultures of variable ages grown on heart broth.*

Experimental details described in the text. The reduction times are given in minutes. In this and subsequent tables the shortest time for each donor is given in heavy type. Temperature 45°. All the bacterial suspensions were standardised to a turbidity $\equiv 5 \times 10^8$ *Bact. coli* cells per ml.

Substrate	Period of growth in hours						Ratio 4 hr./ 24 hr.
	4	6	12	24	48	72	
Control	>120	113	68	>120	>120	>120	—
Formate	2·7	2·9	3·0	2	2	2	1·35
Lactate	8·9	7	3·7	2·9	2·5	3·5	3·07
Glucose	5·2	4·1	3·5	3	23	>120	1·73
Succinate	18·5	14·3	6	3	6	8·3	6·17
Glutamate	37	29	7	5	100	>120	7·4
Alanine	29	20·5	16·5	9·5	>120	>120	3·05
Tryptophan	>120	43	32·5	20	>120	>120	>6
Ratios:							
Lactate/formate	2·93	2·42	1·20	1·45	1·25	1·75	—
Glucose/formate	1·93	1·41	1·17	1·5	11·5	> 60	—
Glucose/lactate	0·58	0·59	0·95	1·03	9·2	> 34	—
Glutamate/formate	13·7	10·0	2·33	2·5	50	> 60	—

pardonable error to conclude that the suspensions were in reality obtained from quite different organisms. Thus the times for the 72-hour suspension suggest that it was derived from an organism possessing active formic, lactic and succinic dehydrogenases but lacking the enzymes for sugars and amino-acids, whilst the results with the 24-hour suspension suggest an organism of a different type, showing marked activity with all the substrates.

Bact. coli grown on caseinogen digest broth.

Another strain of *Bact. coli* was grown similarly on a caseinogen digest medium and the activities of its dehydrogenases were examined. The results obtained are similar in character to those described with heart broth (Table II).

Table II. *Dehydrogenase activities of Bact. coli grown on caseinogen digest for variable periods of time.*

Details as for Table I. $\infty = >180$ min.

Substrate	Period of growth in hours						
	5	10	17	24	48	72	96
Control	∞	∞	63	∞	∞	∞	∞
Formate	2·4	2·3	3·5	2·7	2·5	3·5	6
Lactate	15	10	10·5	7·3	6·3	7·5	17
Glucose	6	5·7	4	7·7	7·7	11	∞
Succinate	11	13	4·5	6·5	9·5	14	58
Glutamate	5·5	6·3	5·6	7	9	11	16
Alanine	112	106	18	84	∞	∞	∞
Leucine	150	146	21	90	95	∞	∞

The formate enzyme again shows the least variation but there are signs of a falling off in its activity after growth for 96 hours, a diminution which is quite definite after 160 hours' growth. With many of the enzymes there are indications of an increasing activity of the dehydrogenases during the early hours of growth.

This variability in enzyme activity was also shown aerobically in the Barcroft differential manometer. Table III illustrates the rate at which oxygen was absorbed by similar *Bact. coli* suspensions in the presence of certain substrates.

Table III. *The rate of oxygen absorption by Bact. coli grown for variable periods on caseinogen digest.*

Experimental details for the preparation of the bacterial suspensions ($1 \text{ ml.} \equiv 5 \times 10^8$ cells) as given in the text. The figures represent the oxygen absorption in $\mu\text{l.}$ at N.T.P., by the various bacterial suspensions in the presence of the named substrates. Both cups of the manometers contained bacterial suspensions and buffers but only the right-hand cup contained the substrate. The usual technique was followed [Dixon, 1934].

Substrate	Period of growth in hours							
	2	5	10	17	24	48	96	160
Formate	80	71	70	72	72	69	—	23
Glucose	80	99	182	169	178	87	42	32
Glutamate	176	178	240	300	238	200	36	45
Alanine	12	19	59	41	142	—	—	20

The activity of the formic enzyme is again remarkably constant but falls off after prolonged growth, whilst the other enzymes examined exhibit more variable activities which at first increase but later diminish in a way similar to that demonstrated anaerobically.

Variation in the dehydrogenase activities of other organisms with the period of growth.

The reduction times obtained with various substrates in the presence of suspensions of *Bact. aerogenes*, *Bact. pullorum* and of *Bact. suispestifer* that have been grown for variable periods of time on nutrient media are displayed in

Table IV. *The dehydrogenase activities of Bact. aerogenes grown on heart broth.*

Details as for Table I. Each Thunberg tube contained 1 ml. of bacterial suspension corresponding in turbidity to a *Bact. coli* suspension containing 7×10^8 cells per ml.

Substrate	Period of growth in hours											
	0	3	6	12	18	25	36	48	72	96	120	144
Control	40	40	45	>120	>120	>120	>120	>120	>120	>120	>120	>120
Formate	4	3.7	7	11.7	11.7	11.3	11.5	14	14	14.3	44	63
Lactate	15	4	5	6.5	6.5	3.3	3	3.5	4	4	9	10.5
Glucose	6	3.7	3.7	6.5	6	5.3	5.5	5.3	9.3	11	120	>120
Succinate	11.5	11	11.3	25	29	9	7.7	10	21	23	>120	>120
Glutamate	6.5	4.7	4	5	5.7	6.5	9	9.5	14	17.5	>120	>120
Tryptophan	>80	24	13	30	30	32	34	—	>120	>120	>120	>120

Table V. *Dehydrogenase activities of Bact. pullorum grown on nutrient agar for variable periods of time.*

Experimental details as for Table I, except that the original suspension was obtained by washing off the bacteria from the solid medium with a few ml. of sterile saline. The suspensions used were equivalent to *Bact. coli* suspensions containing 16×10^8 cells per ml. $\infty = >120$ min.

Substrate	Period of growth in hours							
	5	9	12	18	24	48	72	120
Control	∞	∞	∞	∞	∞	∞	66	∞
Formate	—	∞	90	26	18.5	30	40	—
Lactate	27	21	20	9.7	11.7	4	7	27
Glucose	—	27	18	20	6	4	8.5	13
Succinate	—	—	—	35	8.7	16	9	—
Glutamate	—	29	22	22	13.3	8.5	6.5	26
Glycine	—	—	52	23.3	24.3	100	—	∞
Alanine	—	—	—	60	40	15	29	∞
Tryptophan	—	∞	∞	85	48	∞	—	∞

Table VI. *Dehydrogenase activities of Bact. suispestifer grown on nutrient agar for variable periods of time.*Experimental details as for Table V. The bacterial suspensions contained 20×10^8 cells per ml.

Substrate	Period of growth in hours					
	5	8	12	24	54	72
Control	∞	∞	∞	30	46	∞
Formate	4.6	6.8	5.3	7.2	7.7	29
Lactate	36.5	5	3.5	4.5	5.8	15
Glucose	∞	5.8	4	5.3	8.6	27
Succinate	53	8.5	6.7	9.8	9.5	34
Glutamate	∞	7.5	7.3	7.5	10.1	31

Tables IV, V and VI. The results exhibit a similar variability to those previously obtained with suspensions of *Bact. coli*. With these three organisms the formate enzyme is comparatively weak but it is especially so with *Bact. pullorum*.

DISCUSSION.

It has been shown that the dehydrogenase activity of many, if not all, bacterial cells may vary considerably with the period between inoculation and reaping of the organism. There appears to be a general tendency for the enzyme activities to increase at first and subsequently to decrease. Whether the maximum activity has any relationship to the logarithmic phase of growth cannot be stated at present, although it usually occurs within the first 24 hours of growth. That the variability of dehydrogenase activity is not identical with each enzyme is clearly shown by an examination of the ratios of reduction times given in Table I. The curves in Fig. 1 show that, although many bacteria exhibit varia-

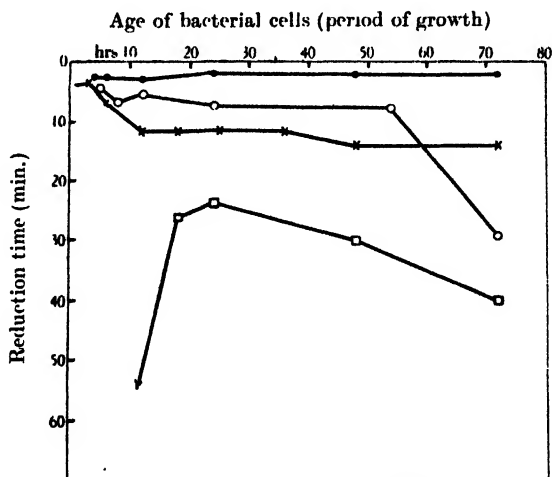


Fig. 1. Variability of the formic dehydrogenase of different organisms. ●—● *Bact. coli* (heart broth); ×—× *Bact. aerogenes* (heart broth); □—□ *Bact. pullorum* (nutrient agar); ○—○ *Bact. suispestifer* (nutrient agar).

tions for particular dehydrogenases (e.g. the formic dehydrogenase), the variations obtained are not necessarily the same with the different organisms. It is not our intention in this paper to assign causes for these variations but several possible factors that might influence the enzymic activity of such cellular suspensions as have been examined may be cited. Thus the activity might be influenced

by the proportion of viable to total cells in the culture medium or in the actual cell suspension used for the tests, by differences in permeability and other properties associated with the "condition" of the cell with respect to its growth phase, by variation in the concentration of co-enzymes, by differences induced in the cell by variations in the chemical or physical nature of the medium during the course of growth or by the effect of these earlier conditions upon the ability of the cells to undergo some change in the Thunberg tube, such as a change in shape or cell division, which change might affect the rate of reduction of the methylene blue. It is hoped to examine the effects of many of these factors in further work, meanwhile it is important to emphasise that the apparent enzymic complex of bacterial suspensions prepared and examined in the way originally described by Quastel and Whetham [1924] does not necessarily indicate a range of activities characteristic of that organism throughout its life (as was more or less implied by Quastel and Wooldridge [1925]) for it appears not only that the order of the activities of the various enzymes will differ among themselves but that certain enzymes which seem to be inactive at one time may be very active at some other stage in the life of the organism. The importance of this fact and its bearing upon the question of "constitutive" and "adaptive" enzymes is obvious. Thus an "adaptive" enzyme may simply be a "constitutive" enzyme which is active apparently only during a part of the life of the cell and is not normally recognised by the technique most usually employed in the enzymic examination of the organism. By suitably altering the conditions of growth of the organism, however, the concentration and stability of such an enzyme may be increased so that it becomes readily detectable by the usual technique.

SUMMARY.

1. It is shown that the activity of most bacterial dehydrogenases may vary considerably with the time the organism is allowed to grow before reaping.
2. The activity of most of these enzymes appears to increase at first, to reach a maximum and subsequently to diminish. This variability is generally least with the formic acid enzyme, *vide* the results with *Bact. coli*, but even with this enzyme it may be great, especially with some other organisms such as *Bact. pullorum*.
3. As the series of reduction times obtained with a standard cell suspension and several substrates may differ considerably with the initial period of growth of the cells of the suspension, it is concluded that to secure a true picture of the enzymic make-up of organisms such as bacteria it is necessary to examine the organism at different stages in its growth.

Our thanks are due to the Medical Research Council for personal grants to two of us (R. K. and V. G.), and for a grant towards the expenditure entailed in this work.

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CXXXV. THE DETERMINATION OF VITAMIN A BY MEANS OF ITS INFLUENCE ON THE VAGINAL CONTENTS OF THE RAT.

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COWARD *et al.* [1935] report on a method of estimating vitamin A by influencing the constitution of the vaginal secretion of rats fed on a vitamin A-free diet. We should like to point out that previous to 1930 we carried out the testing of vitamin A on this principle and published a description of the method [1930, 1]. In the same year this new method of testing was also communicated by us to the Congress for Sexual Research held in London [1930, 2].

The oestrus-like appearances occurring in rats fed on vitamin A-free diet we called "Kolpokeratose". This condition, as we were able to prove, has no connection whatever with the appearance of oestrus, but represents a pathological cornification of the vaginal epithelium and can be quickly caused to disappear by the administration of cod-liver oil or vitamin A. The test based on this appearance we called the "Kolpokeratose test".

Both these expressions, "Kolpokeratose" and "Kolpokeratose test", have been adopted in scientific literature and used in numerous papers appearing later on this test, which confirm its excellent utility for the standardisation of vitamin A. [See Wolff and Oberhoff, 1933; Klussman and Simola, 1933; Moll, *et al.*, 1933; Bomschow, 1935.]

Various groups of workers, notably Moll *et al.* [1933], have endeavoured, with success, to improve the technique of this test.

We wish to draw attention to these facts as the authors do not mention the literature on the "Kolpokeratose test", nor do they even make use of the general expressions, "Kolpokeratose and Kolpokeratose test".

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CXXXVI. SELECTIVE FERMENTATION.

III. FERMENTATION OF HEXOSE-PENTOSE MIXTURES.

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(Received February 28th, 1936.)

THE mutual influence of fermentable hexoses on the fermentation of each has been studied using several varieties of yeast and has been reported in preceding communications [Sobotka and Reiner, 1930, 2, 3]. In the present study, we have investigated the influence of non-fermentable sugars on the fermentation of fermentable hexoses. We measured the rate of carbon dioxide formation from mixtures of glucose or fructose with a pentose and also determined in one series of experiments the total amount of carbon dioxide developed from such mixtures.

It appears that the fermentation by brewer's yeast of glucose, and more so that of fructose, is retarded in the presence of xylose and also of arabinose. This effect was observed in all sugar concentrations tested from 0.8 to 6.7% and with yeast concentrations from 0.6 to 15%, under aerobic and anaerobic conditions. The inhibitory effect of the pentose varied parallel with the ratio of its concentration to that of the hexose, leading sometimes to almost complete suppression of fermentation, particularly with yeasts that had been kept more than 24 hours in the ice-chest. Fructose fermentation was inhibited more than glucose fermentation, and xylose proved a stronger inhibitor than arabinose.

While fermentation went slower in the presence of the non-fermentable pentoses, it progressed further. Thus, the deficit between the actual and the theoretical amounts of carbon dioxide obtained at the end of fermentation was decreased in the presence of xylose, even vanishing in some instances.

In analogous experiments with baker's yeast no such effects on the rate and amount of fermentation could be observed. On the contrary, in the case of aerobic fermentation of glucose by baker's yeast an acceleration of the development of carbon dioxide took place when xylose was present.

In an attempt to explain these observations the possibility that the total sugar concentration reached a level high enough to impair the progress of fermentation could be eliminated. On seeking more specific explanations, competition of the pentose with the hexose in one of the early stages of fermentation had to be considered. Concerning phosphorylation no disappearance of inorganic phosphate could be observed with xylose when using a dry yeast preparation which effectively phosphorylated glucose in parallel experiments.

In order to evaluate the possible influence of diffusion on the "pentose-effect", a study was undertaken of the rate at which various sugars diffuse into the yeast cell. To this end known amounts of yeast were treated with a solution of a given concentration of xylose, arabinose or other unfermentable carbohydrate. Samples of the supernatant, withdrawn at intervals, revealed the rate of disappearance of the sugar from the solution into the yeast cell. This rate of diffusion is rarely high enough to allow an equilibrium to be established. With most of the sugars tested, diffusion was so slow, that no equilibrium was reached

within 18 hours, at which time the yeast had begun to autolyse. But the amount of sugar diffused within a given initial period, *e.g.* one hour, serves well for purposes of comparison. In order to make the diffusion rate of fermentable sugars available for comparison, fermentation had to be suppressed. This was accomplished by means of iodoacetic acid in a dilution sufficient to prevent fermentation but without significant influence on the rate of diffusion as tested with xylose.

According to the speed of diffusion into the yeast cell the compounds tested may be arranged in the following order: xylose > glucose > galactose > *d*-arabinose > *l*-arabinose > mannitol > rhamnose > lactose. The remarkable speed with which xylose is taken up by the cell suggests that this sugar competes with the fermentable hexoses so effectively for entrance into the yeast cell that it may retard their fermentation under certain conditions. The diffusion rates of fructose and glucose were compared in one experiment (no. 85) using yeast that had been treated with iodoacetic acid. The diffusion of fructose was about one-fourth slower than that of glucose, and this difference may be responsible (a) for the faster fermentation of glucose from glucose-fructose mixtures (previous papers), (b) for the greater inhibition of fructose than of glucose fermentation in the presence of xylose and (c) for the moderate inhibitory effect of arabinose on fructose fermentation. The slower diffusion of arabinose as compared with xylose explains why only the latter has a significant effect on glucose fermentation. It has been shown [Nord and Weichherz, 1929] that diffusion is a controlling factor in fermentation, since rapid stirring of fermenting sugar solutions may accelerate the rate of fermentation. Thus, it is likely that competitive diffusion is one of the causes of the "pentose-effect". However, it should be noted that the diffusion measurements yielded analogous results for baker's and for brewer's yeast, but that the pentose inhibition was confined to brewer's yeast.

The great differences in rate of diffusion of various sugars are of interest beyond the explanation of the mechanism of the "pentose-effect" and of selective fermentation. They demonstrate that in the domain of diffusion phenomena small structural and steric differences can cause as high a degree of specificity as one is accustomed to associate with enzymic and immunological reactions. Rate of diffusion is not determined by a simple physical property such as molecular weight or merely by molecular structure, but depends also on steric configuration. Thus, it differs for two substances whose properties are identical except for their opposite optical activities as in the case of *d*- and *l*-arabinose. This confirms our belief that diffusion is based on transient chemical combination with constituents of the cell-wall. The comparison of the diffusion of xylose, arabinose, glucose and galactose shows an analogy between the pair xylose-glucose and arabinose-galactose in regard to the speed of diffusion. As the structural resemblance in each pair issues from the aldehydic group, it can be assumed that this group enters into the chemical reactions with the cell-wall constituents responsible for the selective diffusion phenomena in our experiments.

The study of diffusion of non-fermentable sugars into unicellular organisms can be applied to the differentiation of extracellular, intracellular and chemically bound water in pressed yeast and similar products. At most 90 % of the water contained in yeast, as determined by drying at about 100°, is accessible as solvent for xylose or other sugars entering the cell, and for the less rapidly diffusing sugars this limit is never reached. 10 to 15 % of the water is chemically bound in the cell constituents, especially the plasma protein, and is not available as solvent. On the other hand, with a sugar like lactose which practically does not diffuse into the yeast cell, one may observe an instantaneous disappearance of a few per cent. from the supernatant fluid indicating the free and immediate accessi-

bility of about one-tenth of the water content of the pressed yeast. This is the amount of moisture on the surface of the cells and in the spaces between them and corresponds to the figures for extracellular water obtained by rapid washing with chilled alcohol or acetone. This amount of extracellular water is subject to great variations depending on the degree of pressure used on the yeast, and these variations are reflected in the variations of lactose absorption (*e.g.* Exps. 74 and 75).

The increased yield of carbon dioxide from hexose fermentation in the presence of pentose must be attributed to a hexose-saving effect of the pentose. Whether the pentoses enter any of the side-reactions such as glycerol formation, which are made responsible for the usual carbon dioxide deficit in alcoholic fermentation, or play some part in respiration and formation of polysaccharides and other cell-constituents, by replacing hexoses in other reactions, they release them for alcoholic fermentation. It is likely that such sugar-saving effects of non-fermentable carbohydrates contained in molasses are utilised in industrial fermentations without recognition of the underlying mechanism.

EXPERIMENTAL.

Fermentation of mixtures of a hexose with a pentose.

The experiments recorded in Table I were carried out in the Van Iterson-Kluyver apparatus [Sobotka and Reiner, 1930, 2, p. 928]. The amounts of sugar and of fresh yeast and the volume of solution, which was always pre-saturated

Table I. *Fermentation of mixtures of a hexose with a pentose.*

No.	Hexose mg.	Pentose mg.	Yeast g.	Vol. ml.	Time for half fermentation		Ratio of Rate with pentose Rate without pentose %	Yield of CO ₂	
					Without pentose min.	With pentose min.		Without pentose % of theory	With pentose % of theory
Lager yeast									
1	Glucose 50	Xylose 50	0.2	2.0	81	91	89	—	—
	„ 50	Arabinose 50	0.2	2.0	—	84	96	—	—
	Fructose 50	Xylose 50	0.2	2.0	84	103	81	—	—
	„ 50	Arabinose 50	0.2	2.0	—	98	86	—	—
2	Glucose 50	Xylose 25	0.2	1.5	121	153	79	—	—
	„ 50	Arabinose 25	0.2	1.5	—	135	90	—	—
3	Glucose 50	Xylose 50	0.2	2.0	36	48	75	71	89
	Fructose 50	„ 50	0.2	—	42	53	79	64	76
4	Glucose 30	Xylose 30	0.2	1.3	25	27	93	86	92
	„ 60	„ 60	0.2	1.6	46	51	90	86	88
5	Glucose 30	Xylose 30	0.2	1.3	19	29	65	—	—
	„ 60	„ 60	0.2	1.6	34	40	85	—	—
6	Glucose 50	—	0.2	1.5	47	—	—	93	—
	„ 50	Xylose 100	0.4	3.0	24	30	80	91	97
	„ 50	„ 300	0.8	6.0	<21	23	<90	80	96
7	Fructose 50	—	0.2	1.5	76	—	—	101	—
	„ 50	Xylose 100	0.4	3.0	42	54	78	89	103
	„ 50	„ 300	0.8	6.0	29	46	63	97	107
Baker's yeast									
8	Glucose 50	Xylose 50	0.2	1.0	67	68	98	85	87
	Fructose 50	„ 50	0.2	1.0	71	77	92	86	87
9	Glucose 50	Xylose 100	0.2	1.0	81	80	101	87	87
	Fructose 50	„ 100	0.2	1.0	85	92	92	84	85

Table II. *Rate of fermentation in absence and presence of xylose.*

All fermentations carried out in Barcroft-Warburg manometers at 30° with 16 mg. yeast (ca. 4 mg. dry weight) in 2 ml.

(a. 4 mg. dry weight) in 2 ml.				Ratio of Rate with pentose Rate without pentose %			Remarks	
No.	Glucose %	Xylose %	μ l. CO ₂ developed in 30 min.		Pentose Hexose			
			Without xylose	With xylose	1:1	2:1		3:1
Lager yeast								
10	5.0	5.0	488	548	112	—	—	Anaerobic
	5.0	5.0	447	456	102	—	—	
11	5.0	5.0	364	333	92	—	—	Anaerobic
	5.0	5.0	529	445	84	—	—	
12	6.7	6.7	250	199	80	—	—	
13	5.0	5.0	523	377	72	—	—	
14a	2.5	2.5	137	130	95	—	—	
	—	5.0		105	—	77	—	
15a	2.5	2.5	225	214	95	—	—	
	—	5.0		189	—	84	—	
16a	2.5	2.5	142	116	82	—	—	Anaerobic
	—	5.0		100	—	71	—	
17a	2.5	7.5	67	39	—	—	58	"
18a	2.5	7.5	331	158	—	—	48	—
					92	77	53	Averages
Baker's yeast								
19	2.5	5.0	116	218	—	188	—	
	2.5	5.0	569	601	—	106	—	Anaerobic
20	2.5	5.0	180	273	—	151	—	
21a	2.5	5.0	184	236	—	128	—	
22a	2.5	5.0	187	220	—	118	—	
23a	2.5	5.0	580	599	—	103	—	Anaerobic initial
	2.5	5.0	536	514	—	96	—	Anaerobic after 240 min.
Fructose %								
Lager yeast								
14b	2.5	2.5	143	130	91	—	—	
	2.5	5.0	150	125	83	—	—	
15b	2.5	2.5	197	182	92	—	—	
	—	5.0	277	209	75	—	—	
24	5.0	5.0	365	301	83	—	—	
25	2.5	2.5	254	205	81	—	—	
26	5.0	5.0	464	353	76	—	—	
27	2.5	3.75	157	117	—	75	—	Pent.:Hex. 1.5:1
28	2.5	5.0	189	16	—	8	—	Initial
	2.5	5.0	301	63	—	20	—	After 170 min.
	2.5	5.0	278	118	—	42	—	After 280 min.
29a	2.5	5.0	397	369	—	93	—	
30	2.5	6.25	130	71	—	—	55	Pent.:Hex. 2.5:1
29b	2.5	7.5	397	315	—	—	79	
31	2.5	7.5	288	7	—	—	3	
16b	2.5	2.5	129	122	96	—	—	Anaerobic
	2.5	5.0	125	108	—	86	—	
17b	2.5	7.5	44	19	—	—	43	"
18b	2.5	7.5	325	200	—	—	62	"
					86	71	48	Averages
Baker's yeast								
21b	2.5	5.0	199	225	—	113	—	
22b	2.5	5.0	192	209	—	109	—	
23b	2.5	5.0	610	588	—	96	—	Anaerobic

Experiments numbered 14a and 14b, etc. were carried out simultaneously using samples of the same yeast.

with carbon dioxide, are given in Table I. The theoretical amount of carbon dioxide obtainable from the given amount of fermentable sugar was calculated and corrected for temperature (22° to 27°). The gas burettes were shaken and read every five minutes. The rate of fermentation is expressed by the time in minutes required for half fermentation, *i.e.* for the development of 50 % of the amount of carbon dioxide theoretically possible [Sobotka, 1924]. The samples of baker's yeast used in this and in the following series displayed uniformity in their fermenting power. Greater deviations in rate of fermentation under identical conditions were experienced with the various specimens of brewer's yeast depending on freshness, season and other factors beyond our control. The total solids varied from 25 to 28 %.

The series of experiments recorded in Table II was carried out in the Barcroft-Warburg apparatus, using conical vessels of about 20 ml. capacity in a water-bath kept at 30.0°. The amount of yeast applied was invariably 16 mg., the total volume 2 ml. The velocities given in Table II are initial velocities and are expressed in μ l. carbon dioxide developed during the first 30 min. In some instances, the observations were interrupted after this period, but the fermentation was allowed to continue with the stopcocks opened; readings were then resumed after 2 or 5 hours. At this time, because of the relatively small amount of yeast, the sugar content was far from exhausted and fermentation still proceeded at full speed, the absolute and relative rates not deviating from the initial rates to a significant degree (see Exp. 23*a*), except in Exp. 28, where the fermentation in presence of pentose was almost completely suppressed in the beginning, but increased in the subsequent stages. Regardless of the variations in fermenting power of the Lager yeast, xylose consistently exerted a retarding influence. This effect remained unaltered under anaerobic conditions. On the other hand, in the case of baker's yeast no retardation was observed, but in the fermentation of glucose the rate of carbon dioxide development was frequently accelerated.

Diffusion experiments.¹

Approximately 5 % solutions of the various sugars were prepared and the exact concentration was determined. Fresh yeast was washed twice with distilled water to remove any reducing substances, centrifuged at high speed and pressed. 20 g. of the pressed yeast were then suspended by thorough mixing in 20 ml. of the sugar solution. The amount of sugar varied between 120 and 180 mg. per g. of dry yeast except in Exps. 62*b*, 62*c*, and 70. After the intervals given in Table III aliquot samples of the suspension were withdrawn and centrifuged, and the clear supernatant was analysed for sugar by Hanes's modification of the Hagedorn-Jensen method, using the tables of Sobotka and Reiner [1930, 1]. Mannitol was determined polarimetrically after saturation of the solution with borax. The amount of sugar which had passed into the yeast was calculated from the original amount *minus* the residual sugar content of the supernatant.

The water content of the yeast determined by drying at 100° was made the basis of the calculation of how much sugar could be removed from the supernatant if the dissolved sugar reached the same concentration within as without the cell; *e.g.* when 20 g. of yeast containing 28.7 % total solids (5.74 g.) are added to 20 ml. of sugar solution, the amount of potential solvent is increased to 34.26 ml. In case of an ideal equilibrium 14.26/34.26 parts or 41.7 % of the

¹ A preliminary report on these studies was given at the December Meeting 1933 of the Biochemical Society, London.

Table III. *Diffusion of various sugars into the yeast cell.*

20 ml. of approximately 5% sugar solutions were mixed with 20 g. yeast (total solids 2 to 3%). From these data we calculated in each instance the amount of sugar that would disappear from the supernatant if equilibrium of the sugar concentration within and without the cell were established. The figures given in the table are the percentages of this amount which have actually disappeared from the supernatant at a given time.

Sugar		Diffusion after			
		15 min.	1 hr.	2 hrs.	18 hrs.
Experiments with brewer's yeast (Lager)					
51	Xylose	88.4	—	—	After 5 min. —
52	Galactose	69.6	—	84.5	—
53a	d-Arabinose	44.3	73.2	—	—
53b	l-Arabinose	33.9	44.4	—	—
54a	d-Arabinose	31.1	67.2	—	—
54b	l-Arabinose	25.8	38.7	—	—
55	Rhamnose	20.4	31.2	—	—
56	Mannitol	30.2	—	—	—
57a	α -Lactose	14.6	20.7	—	—
57b	β -Lactose	16.5	22.7	—	—
58	Lactose	13.4	22.4	—	Courtesy Milk Sugar Institute
59	Lactose	15.8	—	—	—
Experiments with baker's yeast (Fleischmann)					
60	Xylose	76.5	—	—	After 5 min. —
61	Xylose	83.8	—	—	—
62a	Xylose	82.8	—	—	—
62b	Xylose	80.2	—	—	From a 10% xylose solution
62c	Xylose	75.9	—	—	From a 20% xylose solution
63	Glucose	61.0	—	—	After 5 min. —
64	Glucose	95.4	—	—	Beginning fermentation?
65	Maltose	51.1	—	—	28.9% after 5 min. —
66	Galactose	26.0	—	67.7	—
67	Galactose	—	24.9	65.8	81.7 —
68	d-Arabinose	18.7	—	31.0	—
69a	d-Arabinose	—	38.1	67.7	91.1 —
69b	d-Arabinose	—	29.9	55.2	88.4 —
69c	l-Arabinose	—	22.1	24.3	52.3 —
70	d-Arabinose	—	69.8	73.6	94.3 —
71	Rhamnose	24.2	33.0	—	—
72	Mannitol	—	33.3	—	—
73	Mannitol	17.0	23.2	—	—
74	Lactose	9.6	20.3	—	—
75	Lactose	—	—	9.1	—
Extra dry yeast (total solids 34%)					
Baker's yeast treated with iodoacetic acid 0.4%					
76	Xylose	83.2	—	—	—
77	Xylose	71.2	82.0	—	—
78	Xylose	69.7	—	—	—
79	Xylose	67.5	77.2	—	—
80	Xylose	—	88.5	88.5	90.8 —
81	Glucose	61.3	—	—	—
82	Glucose	44.7	53.4	—	—
83	Glucose	44.1	—	—	—
84	Glucose	34.8	56.7	—	—
85a	Glucose	—	45.4	55.1	81.2 —
85b	Invert sugar	—	56.9	61.1	85.9 —
85c	Fructose	—	34.0	42.5	62.0 —
Experiments with <i>Saccharomyces marxiianus</i>					
86	Maltose	67.0	—	—	—
87a	Glucose	51.8	—	—	Fermentation suppressed with 0.4% CH ₂ ICOOH
87b	Maltose	32.8	—	—	—

dissolved sugar, *e.g.* 401 mg. out of 963 mg. would disappear from the supernatant, provided that under the conditions of the experiment the volume of the yeast cells remains constant; this was ascertained in control experiments. As an additional check, the yeast, after having taken up a known amount of a non-fermentable sugar, was separated and re-suspended in a given amount of distilled water: thus, we could demonstrate that the sugar would diffuse from within the cell to the outside in the expected measure when the gradient of concentration was reversed.

The yeast samples used in Exps. 76–85, and 87, were treated for 15 min. with an equal amount of an aqueous 0.4% solution of iodoacetic acid, which was sufficient to suppress any subsequent fermentation.

The inability of some yeasts to ferment certain carbohydrates, and on the other hand, the adaptation of some strains of yeast to unaccustomed sugars remain to be investigated from the viewpoint of selective diffusion. The inability of *Saccharomyces marxianus* to ferment maltose [Sobotka and Reiner, 1930, 2] is due to other factors, as diffusion of maltose in this yeast occurred at normal speed (Exp. 86), more than half of the rate of glucose diffusion (Exp. 87, *cf.* Exps. 63 and 65).

Selective diffusion. In a few instances an attempt was made to compare the rates of diffusion of two components of a sugar mixture. In experiments on the simultaneous diffusion of *d*- and *l*-arabinose and of glucose and fructose it was found that the *l*-(+)-arabinose diffuses half as rapidly only as its *d*-(-)-isomeride into the yeast. In the case of invert sugar, the glucose molecules penetrate the cell wall at about 1.33 times the rate of those of fructose. Table IV illustrates the

Table IV. *Diffusion of sugar mixtures.*

Time hrs.	Outside solution		Outside solution sugar partition ‰		Intracellular sugar partition (calculated) ‰	
	‰ sugar	$[\alpha]_D$	<i>l</i> -Arabinose	<i>d</i> -Arabinose	<i>l</i> -Arabinose	<i>d</i> -Arabinose
No. 69 <i>b</i>						
0	5.28	0	50.0	50.0	—	—
1	4.64	+ 5.2°	52.5	47.5	31.2	68.8
2	4.10	+ 7.3	53.5	46.5	38.1	61.9
18	3.40	+ 3.5	51.7	48.3	46.8	53.2
No. 70						
0	2.59	0	50.0	50.0	—	—
1	1.80	+ 13.3	56.3	43.7	36.1	63.9
2	1.76	+ 19.3	59.2	40.8	30.7	69.3
18	1.54	0	50.0	50.0	50.0	50.0
No. 85 <i>b</i>						
			Fructose	Glucose	Fructose	Glucose
0	4.62	- 19.0	49.5	50.5	—	—
1	3.52	- 19.9	50.1	49.9	47.6	52.4
2	3.44	- 22.8	52.1	47.9	41.9	58.1
18	2.96	- 18.0	49.0	51.0	50.4	49.6

manner of calculation of the relative speeds of diffusion in Exps. 69*b*, 70, and 85*b*, where the supernatant was analysed polarimetrically for the individual components in addition to the determination of the total sugar content.

SUMMARY.

1. Pentoses, particularly xylose, have a retarding effect on the rate of fermentation of hexoses by brewer's yeast; however they sometimes increase the total amount of CO₂ evolved.

2. The rates of diffusion into the yeast cell of a number of fermentable and non-fermentable sugars differ to a considerable and significant degree. Competitive diffusion is a major factor in the mechanism of the "pentose-effect" and of selective fermentation.

3. Studies of diffusion permit the differentiation of extracellular, intracellular and chemically bound water in yeast.

We wish to thank Mr Leo Wallerstein for numerous samples of brewer's yeast, Dr Chas. N. Frey and the Manufacturing Department of Standard Brands Inc. for specially pressed baker's yeast, and Mr R. T. Baldwin of the Milk Sugar Institute for samples of purest lactose and of β -lactose.

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- (e) *Hydrochloric acid*, 1%.
- (f) *Potassium chloride*, 25% in 0.2% HCl.
- (g) *Cuprous chloride*. Immediately before using 0.3 g. of well-powdered cuprous chloride is washed in 25 ml. of 1% HCl, allowed to settle and after decantation of the supernatant liquid dissolved in 2 ml. of the KCl solution.
- (h) *Uric acid reagent* [Folin, 1934].

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² Fellowship maintained by the Women's Auxiliary of the Lankenau Hospital Research Institute.

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must be kept cold and
may be used throughout

qual vols. of 2 *M* sodium

10 vols. of 2 *M* sodium

Technique.

To 8 ml. of the standard cystine or cysteine solution in a graduated 15 ml. centrifuge-tube were added 5 ml. of buffer at p_H 4.6 and 10 to 12 drops of the freshly prepared cuprous chloride solution. The mixture was kept for 30–40 min., centrifuged for 5 min. at moderate speed and the supernatant liquid decanted. The precipitate was stirred with a fine glass rod in buffer at p_H 5.2 added to the 8 ml. mark, after which H_2S was bubbled through for 3–4 min. with constant stirring to break up any particles of the mercaptide. The solution was filtered through a small fine filter-paper into a test-tube and nitrogen or carbon dioxide gas bubbled through vigorously for about 20 min. to remove H_2S . A 5 ml. aliquot part was pipetted into a 25 ml. volumetric flask containing 1.5 ml. of buffer at p_H 5.2, followed by 2 ml. of the uric acid reagent. At the same time 6.5 ml. of the same buffer were added to 2 ml. of the standard cysteine solution in a second 25 ml. volumetric flask and 2 ml. of the uric acid reagent added. Both solutions were made up to volume with water and read in a colorimeter after 20 min.

$$\frac{\text{Cystine standard in mol.}}{2} \times \frac{20}{R} = \text{mol. cystine recovered.}$$

Mercuric chloride precipitation.

Solutions. In addition to solutions *a*, *b*, *d* and *h* listed above, 2*M* sodium acetate solution and a saturated solution of mercuric chloride were employed.

Procedure. The same procedure as in the cuprous chloride precipitation was followed, except that 5 ml. of 2*M* sodium acetate were added to the standard solution, followed by 2 ml. of saturated $HgCl_2$. Since precipitation is slow, the mixture was kept several hours, usually overnight, after which the tubes were centrifuged and the precipitate treated as described above.

Vickery and Leavenworth [1930], upon decomposing their silver mercaptide with H_2S , found 97% of the nitrogen in the form of cysteine and 3% in the form of cystine. In the experiments reported in this paper, repeated attempts were made to demonstrate the presence of disulphide in the solution after treatment of the copper or mercury precipitate with H_2S and aeration of the filtrate with CO_2 . In no case did the addition of sodium bisulphite to the test solution before

Table I. *Recovery of cystine and cysteine as cysteine after precipitation with Cu_2Cl_2 under varying conditions.*

Cystine 0.001 <i>M</i> ml.	Cysteine 0.002 <i>M</i> ml.	Cu_2Cl_2 drops	Time ppt. stood min.	Recovery in % of 5 ml. aliquot			No. tests
				Max.	Min.	Av.	
8	—	10–12	40	97.7	88.0	93.5	30
—	8	10–12	40	98.3	87.3	94.4	25
Effect of varying the quantity of cystine or cysteine.							
2	—	10	40	98.3	87.2	93.0	6
4	—	10	40	97.0	89.0	92.3	6
—	2	10	40	97.0	90.1	92.5	6
—	4	10	40	98.1	87.4	91.3	6
Effect of varying the amount of precipitant.							
8	—	5	40	91.6	85.0	87.2	7
8	—	8	40	94.2	84.6	93.1	10
8	—	15	40	77.3	74.2	75.1	4
—	8	15	40	—	—	—	—
Effect of standing for varying periods after precipitation.							
8	—	10–12	0	90.5	82.5	86.9	5
—	8	10–12	0	93.8	72.2	83.6	8
8	—	10–12	20	95.8	84.9	93.2	8
—	8	10–12	20	96.8	87.3	95.0	8
8	—	10–12	120	97.0	87.2	93.0	6
—	8	10–12	120	97.7	86.4	95.7	6

introduction of the uric acid reagent bring about development of more than twice the colour produced with the uric acid reagent alone, as should theoretically have occurred if mixtures of cystine and cysteine instead of cysteine alone had been present.

The results upon varying the conditions of the precipitation of cystine and cysteine and their recoveries as cysteine are reported in Tables I and II.

Table II. *Recovery of cystine and cysteine as cysteine after precipitation with HgCl_2 under varying conditions.*

Cystine 0.001 <i>M</i> ml.	Cysteine 0.002 <i>M</i> ml.	Medium			HgCl_2 ml.	Recovery % theoretical range	No. tests
		2 <i>M</i> NaAc ml.	2 <i>M</i> HAc ml.	<i>M</i> NaOH ml.			
5	—	5	—	—	1	32.9-47.3	3
5	—	5	—	—	2	65.9-71.1	30
5	—	5	—	—	3	63.7-70.6	5
5	—	5	—	—	4	62.6-71.2	5
5	—	5	—	—	6	60.3-71.4	5
5	—	5	—	0.2	1	40.0	1
5	—	5	—	0.2	2	42.9-48.9	7
5	—	5	—	0.2	3	43.0-62.7	5
5	—	5	—	0.2	4	49.6	1
5	—	5	—	0.2	6	49.0-56.3	4
5	—	5	1.5	—	—	60.2-65.8	5
—	5	5	—	—	1	80.1-82.0	3
—	5	5	—	—	2	88.0-95.0	10
—	5	5	—	—	4	86.8-90.0	3
—	5	5	0.2	—	2	Reading inconstant	3
—	5	5	0.2	—	4	Reading inconstant	3

Results.

(a) Cu_2Cl_2 .

Recovery under standard conditions. The average yield of cystine from cystine was 93.5% in thirty tests (88-97.7%). The recovery of cysteine from cysteine was not significantly greater, averaging 94.4% (87.3-98.3%).

Effect of varying the quantity of the precipitant. When 5 drops of the cuprous chloride solution were added, the recovery was slightly lower. The failure of the precipitate to settle out promptly indicated that an insufficient amount of the precipitant had been used. When 15 drops of the cuprous chloride were added, the recovery again was low, possibly because of the solubility of the mercaptide in the large amount of potassium chloride added.

Effect of varying the quantity of cystine and cysteine. Since the presence of excess precipitant affects the solubility of the precipitate, the recovery of smaller amounts of the test solutions under otherwise standard conditions was studied. Aliquot parts of 2 and 4 ml. of cystine or cysteine were recovered equally well as 8 ml.

Optimum period for allowing the precipitate to stand before centrifuging. When the precipitate was centrifuged immediately after the addition of cuprous chloride the average recovery fell to 87%. With a delay of 20 min. the average recovery was about the same as with the 40-min. interval, but as the individual values were somewhat erratic, the shorter period was deemed hardly sufficient. Keeping the precipitate overnight did not further increase the yield.

(b) $HgCl_2$.

The yield of cysteine from cystine precipitated with mercuric chloride was lower than when cuprous chloride was used as the precipitating agent (Table II), 69.0% as average under optimum conditions and 71.1 and 65.9% as maximum and minimum respectively. 1 ml. of the mercuric chloride proved insufficient for the precipitation of the amount of cystine employed, and 6 ml. did not further increase the yield. In more acid or alkaline media the yields were lower and greater amounts were determined in the filtrates. The recovery of cysteine was comparable with that of cysteine under the cuprous chloride precipitation.

Effect of varying the temperature. Since it was shown by Vickery and Leavenworth [1930] that, in the silver precipitation, maintaining the solution at 10° or lower tends to prevent decomposition of the mercaptide, the cuprous and mercuric precipitations were carried out at 2°, the reagents being embedded in ice or allowed to stand in a refrigerator at that temperature. Under these conditions the copper mercaptide separated in colloidal form and could not be successfully centrifuged even after 12 hours. The yield averaged about 12% lower than at room temperature. The yield from cysteine was only slightly higher. In the case of the mercury precipitation, the same colloidal effect was not produced, but the yield was not increased above that reported in Table II. Slight improvement in the yield occurred when cysteine was precipitated, the average value being about 95% as against 94% at room temperature. The range of values however was essentially the same, so it was concluded that this difference was not significant.

DISCUSSION.

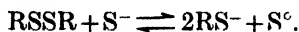
The average recovery of about 95% of the cystine precipitated with cuprous chloride is in close agreement with the figures recorded by Vickery and White [1933], whose recovery averaged 94.6% and who employed cuprous oxide as the precipitant, after preliminary reduction of the cystine to cysteine by tin and acid. They believed this preliminary reduction to be essential for a nearly quantitative yield, as it had been previously demonstrated that the reduction of cystine to cysteine by tin is quantitative, whereas in the reduction which takes place during the precipitation by mercury and silver salts, one-sixth of the cystine had been reported to be oxidised to cysteic acid [Vickery and Leavenworth, 1930; Preisler and Preisler, 1930; Simonsen, 1931-32]. The findings of the present authors that the percentage of cystine recovered without previous reduction is as great as that of cysteine, corroborating the conclusions of Rossouw and Wilken-Jorden, indicates that this simultaneous oxidation and reduction does not take place when cuprous chloride is used as the precipitant under the conditions of these experiments.

Rossouw and Wilken-Jorden, on the basis of 100% recovery of cystine after cuprous chloride precipitation, postulate that each mol. of cystine gives rise to 2 mols. of cysteine as mercaptide instead of 5 from 3 of cystine. According to them the excess cuprous chloride used in the precipitation drives the reaction to the SH side, while in the process of dissolving the mercaptide in dilute acid, the initial state of the cystine is recovered owing to the presence of excess of the cupric ion in the complex molecule. It may be expressed:



and is forced in either direction depending upon whether cupric or cuprous copper is in excess. A second reduction of S—S to SH must be postulated during the subsequent treatment with H_2S .

Andrews and Wyman [1930] rejected the possibility of the reduction by H_2S since they believed that in this event free sulphur would appear in the solution according to the equation:



The authors are able to confirm their observation that no free sulphur appears in these solutions. It is known, however, that the rapid reduction of cystine to cysteine by H_2S does occur in the presence of ions of the heavy metals [Mauthner, 1901], which were not present in the solution tested by Andrews and Wyman. It is reasonable therefore to assume that in the transfer of electrons the copper ions are involved and that the reaction is more complex than these authors suggest. The markedly different results obtained under the mercuric chloride treatment lead to the conclusion that a different set of reactions must be occurring when cystine is being precipitated by the mercuric ion. Here, a yield of 40–75 % of the original cystine is obtained from the precipitate according to the conditions of the experiment, whereas cysteine may be recovered to about the same extent as when cuprous chloride is used. Since the fundamental difference between these two procedures consists in the substitution of the mercuric ion for the strongly reducing cuprous ion, the conclusion seems justifiable that in this case simultaneous oxidation and reduction occur in some such manner as that suggested under the first equation above, and that the reaction goes to completion because of the insoluble character of the mercury mercaptide which is one of the reaction products. At no time, however, could a value as great as 83 % of the original cystine be obtained from the combined precipitate and filtrate, the actual figures varying between 68 and 74 %. Hence some other reaction is possibly also involved.

Since cystine, precipitated by cuprous chloride, could be recovered only to about 95 % on the average following decomposition of the mercaptide with hydrogen sulphide, an attempt was made to ascertain at what step the loss occurred, by substituting decomposition by potassium thiocyanate, as employed by Rossouw and Wilken-Jorden. The following modification of their method was made.

The cuprous precipitate in the centrifuge-tube is stirred into 1 ml. of 0.5 *N* HCl and 1 ml. of 0.1 *N* acetic acid until all particles have disappeared. Water is added to about 5 ml. followed by 0.4 ml. of 10 % KSCN to precipitate cuprous ions and after 2 min. of vigorous stirring, 2 drops of pyridine are added from a finely pointed pipette to precipitate cupric ions. The volume is adjusted to the 8 ml. mark with water and the solution filtered through a fine filter-paper. No SH can be demonstrated at this stage, as stated by Rossouw and Wilken-Jorden. A 5 ml. aliquot part of the filtrate is pipetted into a 25 ml. volumetric flask followed by 6.5 ml. of sodium acetate-acetic acid buffer at p_{H} 5.2, 1.5 ml. of 0.1 *M* NaHSO_3 and 2 ml. of the uric acid reagent. A white precipitate is formed by the excess KSCN and the uric acid reagent. If this precipitate is large in amount, additional reagent must be added. The solution is centrifuged after 15 min. and compared with a cysteine standard prepared 20 min. previously.

The error of a single determination by the phospho-18-tungstic acid method with the filtrate as prepared above is somewhat greater than with the filtrate from the H_2S method, i.e. the proportionality between colour developed and amount of cystine present is less strict, probably owing to the varying amount of the uric acid reagent remaining in the solution after partial precipitation. In all cases, however, the final readings indicated a recovery of 100 % (100 ± 1.5 %).

The filtrate obtained after either method of precipitation may also be used with the Sullivan method as modified by Rossouw and Wilken-Jorden [1934].

In this case however the proportionality between initial amount of cystine and amount recovered was even less strict in the hands of the present authors than with either procedure described above, although recovery of approximately 100 % is indicated. Table III gives a comparison of results by the three methods.

Table III. *Comparison of quantitative determinations of cystine following its precipitation with Cu_2Cl_2 and its subsequent recovery. (a) Decomposition of mercaptide with H_2S and determination with the uric acid reagent. (b) Decomposition of the mercaptide with KSCN and determination with the uric acid reagent. (c) Decomposition of the mercaptide with KSCN and determination by the Sullivan method. In each case results of 5 tests were averaged and the recovery expressed in percentage.*

Cystine pptd.		Standard		Method	Recovered %
0.001 M ml.	0.005 M ml.	0.001 M ml.	0.005 M ml.		
5	—	5	—	(a)	96.3 ± 0.4
4	—	5	—	(a)	94.9 ± 0.5
3	—	5	—	(a)	96.2 ± 0.4
2	—	5	—	(a)	93.9 ± 0.5
1	—	5	—	(a)	94.2 ± 0.5
5	—	5	—	(b)	100.1 ± 0.6
4	—	5	—	(b)	100.2 ± 0.5
3	—	5	—	(b)	98.3 ± 3.4
2	—	5	—	(b)	101.3 ± 4.1
1	—	5	—	(b)	102.0 ± 6.8
—	5	—	5	(c)	100.3 ± 2.3
—	4	—	5	(c)	105.8 ± 3.8
—	3	—	5	(c)	109.6 ± 4.9

These results indicate that the loss of 5 % of the initial cystine in the cuprous chloride and hydrogen sulphide method occurs during the decomposition of the mercaptide by H_2S and the subsequent reduction of the cystine to cysteine. The following experiments were performed: 8 ml. of 0.001 M cystine were introduced into a test-tube, 2 drops of 5 % cupric chloride added and H_2S bubbled through for 0.5 min., then removed with CO_2 . Recovery of cystine from a 5 ml. aliquot part was 100 %, of which about 98 % was cysteine and 2 % cystine. When H_2S was bubbled for 1 min., 100 % was recovered as cysteine. The same experiment was performed with mercuric instead of cupric chloride and similar results were obtained. Since the loss of 5 % of the initial cystine cannot be accounted for in any of these steps, it seems most probable that it occurs by occlusion of cysteine on the copper sulphide. Attempts were made to recover a greater amount by washing the copper sulphide in hot buffer solution, but the yield was increased only by about 1 %.

SUMMARY.

A study has been made of the precipitation of cystine and cysteine from solutions by mercuric chloride and cuprous chloride and its recovery as cysteine in sodium acetate-acetic acid buffer solutions suitable for subsequent use in quantitative determination by phospho-18-tungstic acid.

Recovery following decomposition of the mercury mercaptide by hydrogen sulphide averaged 69 % of the original cystine. Recovery from cuprous mercaptide by the same method averaged 95 % of the original cystine.

Recovery of cystine from cuprous mercaptide by precipitation of the copper with potassium thiocyanate and pyridine yields 100 % of the cystine, but the

filtrate does not lend itself to accurate quantitative determinations by phospho-18-tungstic acid.

The filtrates obtained by either procedure may be used also with the Sullivan method of cystine determination. The proportionality is still less strict than with the phospho-18-tungstic acid method.

The procedures described above, except for the less sensitive Sullivan test, may be carried out with 8 ml. or less of the solution to be tested, containing as little as 1×10^{-5} g. mol. of cystine (0.24 mg.) in the sample. This value, as will be shown in a later paper, is comparable with those found in normal urine.

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CXXXVIII. ULTRACENTRIFUGAL AND ELECTROPHORETIC STUDIES ON THE MILK PROTEINS.

I. INTRODUCTION AND PRELIMINARY RESULTS WITH FRACTIONS FROM SKIM MILK.

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(Received March 30th, 1936.)

Few ultracentrifugal studies on milk proteins have been carried out since the ultracentrifuge was constructed by Svedberg.¹ In 1924 Svedberg and Fåhræus [v. Svedberg, 1930] estimated the degree of dispersion of calcium caseinogenate in cow's milk and concluded that the Ca caseinogenate formed a very polydisperse suspension in the milk serum, that the distribution curve was very flat and that the diameters of the particles were of the order of magnitude of 20–140 $m\mu$ ($m\mu = 10^{-7}$ cm.). The experiments were carried out in relatively low centrifugal fields of about 1500 times gravity.

Svedberg *et al.* [1930, 1, 2] studied purified caseinogen in centrifugal fields up to 100,000 times gravity. They concluded that caseinogen is a very unstable protein and that the molecular weight and the homogeneity of caseinogen depend on the way in which it has been prepared. The bulk of the crude caseinogen prepared by the method of Van Slyke and Baker had a mol. wt. between 75,000 and 100,000. The caseinogen prepared by the Hammarsten method was still more heterogeneous, but it was possible by means of acidified 70 % alcohol to extract from the Hammarsten caseinogen a homogeneous protein with mol. wt. about 375,000.

Svedberg and Sjögren [1930] studied the behaviour of lactalbumin in the ultracentrifuge in fields up to 100,000 times gravity. They found that lactalbumin prepared according to the method of Wichmann was heterogeneous with regard to mol. wt. (12,000–25,000); they mentioned that Tiselius had found it to be electrochemically heterogeneous with isoelectric point near p_H 5.2. Furthermore, they made some experiments in order to ascertain whether the heterogeneity was caused by the procedure of isolation and concluded that the "purified" lactalbumin does not exist in the milk but is formed during the process of "purification", especially by the action of ammonium sulphate in high concentration. According to these authors lactalbumin is formed by successive aggregation from a substance with a mol. wt. not exceeding 1000.

Nichols *et al.* [1931] studied the influence of preheating on the dispersity of Ca caseinogenate in skim milk. They concluded that the maximum on the distribution curve is somewhat more pronounced in the case of preheated skim milk. Most of the particles are less than 200 $m\mu$ in diameter and the mean size is about 90 $m\mu$.

In the autumn of 1934 the author started to investigate the crystalline globulin prepared by Palmer [1934] from the albumin fraction of cow's milk. It was immediately evident that this lactoglobulin was a quite uniform substance

¹ For recent review of the method, see Svedberg [1934, 1, 2, 3, 4].

having a definite mol. wt. and a definite isoelectric point. Prof. Svedberg suggested that the problem of lactalbumin should be taken up again, as the efficiency of the centrifuge had been increased very much since the earlier work in 1930.

In the meantime (spring 1935) Philippi¹ made some experiments in order to find the substance of low mol. wt. expected to form the pre-lactalbumin and in order to investigate details of the association process. Contrary to expectation he found that the proteins which are generally called "lactalbumin" are present in the milk at least in a very early stage of the preparation.

The first part of Philippi's investigation was carried out using the light absorption method. However, this method was found to be unsatisfactory for solving this special problem and the refractometric method worked out by Lamm [1928; 1929] was applied.

By this technique it was shown that skim milk from which caseinogen has been removed by isoelectric precipitation contains three distinct proteins, α , β and γ , which are characterised by different sedimentation constants. The magnitude of these sedimentation constants increases in the order from α to γ . With the available resolving power of the ultracentrifuge it was not possible to detect other components in any appreciable amount.

Values of the sedimentation constant of the α -component between 1 and $2 \cdot 10^{-13}$ were observed, very low values for a protein. The sedimentation constant of the β -component was found to be $3 \cdot 13 \cdot 10^{-13}$ in the p_H range $5 \cdot 5$ – $7 \cdot 0$, identical with that of Palmer's lactoglobulin [Pedersen, 1936] within the limits of error. Hence Philippi concluded that the β -component is identical with the Palmer protein. For the sedimentation constant of the γ -component values between $7 \cdot 0$ and $7 \cdot 4 \cdot 10^{-13}$ were observed.

In the autumn of 1935 Kekwick¹ succeeded in isolating the protein with the low sedimentation constant. The experiments show that it is an electrochemically homogeneous substance: from centrifugal and diffusion experiments which have been carried out so far it seems to be of uniform size.

About the same time the author began to study the sedimentation of skim milk and fractions obtained from it by treatment in different ways. The results of these experiments serve as an introduction to a more detailed study of the high molecular substances present in milk. These experiments serve to show how the Svedberg ultracentrifuge can be used for studying biochemical solutions in their native state, and the changes caused by the different fractionation procedures can be followed.

First there will be given a description of some of the recent modifications of the refractometric method in the sedimentation velocity and the sedimentation equilibrium methods. Finally the experiments carried out on milk will be described.

EXPERIMENTAL TECHNIQUE.

The sedimentation velocities were determined in the same ultracentrifuge as described previously by McFarlane [1935]. For recording sedimentation two different methods have been used, *viz.* the light absorption method and the refractive index method of Lamm [1928; 1929].

The light absorption method. Details of this method may be found in some of the earlier papers of Svedberg *et al.* [*e.g.* 1926 and 1932]. Throughout this work the short wave ultraviolet light absorption of the proteins was used. In order to reduce the influence of the change in the latent image between exposure and

¹ Results not yet published.

development, the photographic plates were stored 1 day before being developed, since the change in the latent image is most pronounced during the first hours after exposure.

Dilute solutions of potassium chromate in 0.05 *M* sodium carbonate solution were used to give the relationship between concentration and blackening on the photographic plate [Svedberg and Eriksson, 1932].

The refractive index method. This method, described some time ago [McFarlane, 1935], was used with the modifications given below.

The projection system. The projection system was improved to give a more linear projection of the scale. In the first projection apparatus (I) used by McFarlane the projection lens was a single Zeiss Tessar with a focal length of 13.5 cm. and an aperture of $F/4.5$; this lens however is not symmetrical, it does not give a linear scale image. By substituting two special Zeiss Tessars as selected for stereoscopic photography ($f=26$ cm. and an aperture of $F/6.3$) one gets a symmetrical projection lens that gives a much more uniform scale. This projection system is called (II). But even this projection system was found to be unsatisfactory, especially when used with some of the new centrifuge cells having a high sedimentation column. Therefore a new projection system (III) has just been constructed. In this case the projection lens is composed of two Zeiss Tessar lenses with focal lengths 36 cm. and an aperture $F/6.3$. This system seems to give as good delineation as is necessary.

The reference scale. In the work of McFarlane [1935] the standard undeviated scale was obtained before the actual experiment by bringing the centrifuge to the desired speed with the cell filled with water and a photograph was taken shortly after this speed was reached. In this work the cells were filled to exactly the same height with the same "solvent" as the one in which the protein was dissolved. By "solvent" is meant the buffer solution of the same concentration as that in the protein solution (or else the ultrafiltrate from this solution). It was found [Pedersen, 1934] that the sedimentation of the small inorganic molecules gives rise to a refractive index gradient and therefore to a deviated scale. Considering the time for establishment of the equilibrium under the conditions used, which is generally from 8 hours to 1 day, it means that even in the absence of a high-molecular substance from the cell one obtains a different displacement of the lines at different times from the start. Therefore throughout this work special reference scale experiments have always been carried out; exposures are taken at short intervals for all the scale distances used; the experiments are extended over the same time interval as used in the actual protein experiment. When the same "solvent" and the same cell are used in all experiments as far as possible it is found that very often the same reference scale can be used for several experiments.

Since the displacements of the lines are proportional to the scale distance and the thickness of the cell, it can be seen that the necessity of taking reference scales becomes greater when thicker cells and longer scale distances are used. In this work nearly all the experiments were carried out with 6 and 12 mm. cells and with scale distances up to 120 mm. (160 mm. in a few special cases). McFarlane generally worked with a 2 mm. cell and scale distances from 20 to 40 mm., so that in his case it was not so necessary to take the same precautions with the reference scale. Some of his "base line" troubles may have been caused by the fact that he used pure water for reference scale experiments instead of the proper salt solution.

The concentration of the sedimenting substance. McFarlane [1935] generally found that the concentration of the sedimenting substance calculated from the

area below the curve was always lower by 10–15% than the analytical value. Some experiments done by the author have shown the same to be the case for horse serum albumin. But for lactoglobulin the value from the sedimentation diagram agreed very well with the analytical value. We therefore believe that the concentration calculated from the curves gives the correct value for the sedimenting substance [Pedersen, 1936].

Sedimentation equilibrium. The concentration gradient in the centrifuge cell was in this case determined in most of the experiments by means of the refractive index method [Lamm, 1928; 1929]. In a few experiments the new refractive index method of Lamm [1933] was used.

CALCULATIONS OF RESULTS.

Sedimentation equilibrium. According to Svedberg [1925; 1926] the molecular weight of a solute may be obtained from its sedimentation equilibrium by means of the equation

$$M = \frac{2RT \ln \frac{c_2}{c_1}}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)} \quad \dots\dots(1),$$

where M = molecular weight, R = gas constant, T = absolute temperature, c_2 and c_1 are the concentrations of the solute at the distances x_2 and x_1 from the axis of rotation, ρ the density of the solution, V the partial specific volume of the solute and ω = angular velocity.

When the light absorption method is used, the *concentrations* at all points along the cell are obtained and this equation may be used directly to calculate the molecular weight of the solute. By the refractive index method the values for the *concentration gradient* at all points along the cell are obtained. According to Lamm [1928; 1929] the scale displacement Z is determined by the equation

$$Z = Gab \frac{dn}{dx} = Gabx \frac{dc}{dx} \quad \text{or} \quad \frac{dx}{dc} = \frac{Z}{Gabx} \quad \dots\dots(2),$$

where G = camera magnification of the displacement, a = the cell thickness, b = optical distance between the scale and the middle of the cell, $\frac{dn}{dx}$ = the refractive index gradient in the cell and $\alpha = \frac{dn}{dc}$ or the refractive increment of the solute.

When the concentration gradient is known at any point in the cell, it is possible to calculate the concentrations throughout the cell, if we assume that the whole material present at the start is still present in solution in the cell when equilibrium is established. That means in the case of a rectangular cell that $\int_{x_0}^{x_n} c_0 dx = \int_{x_0}^{x_n} c_x dx$ where c_0 is the original concentration of the solution and c_x the concentration in the level x , from the centre of rotation after equilibrium is established. In order to find the values for c_x we may use the following procedure.

If the values for $\frac{dc}{dx}$ vary only slightly with x , then $\frac{dc}{dx}$ is calculated for every 0.05 cm. in the cell and as a first approximation it is assumed that at the point x_m (somewhere in the middle of the cell) the concentration $c_m = c_0$ and at the next point $x_{m+1} = x_m + 0.05$ cm., the concentration is (Fig. 1)

$$\begin{aligned} c_{m+1} &= c_0 + \frac{0.05}{2} \left[\left(\frac{dc}{dx} \right)_m + \left(\frac{dc}{dx} \right)_{m+1} \right] \\ c_{m+2} &= c_{m+1} + \frac{0.05}{2} \left[\left(\frac{dc}{dx} \right)_{m+1} + \left(\frac{dc}{dx} \right)_{m+2} \right] \\ c_{m-1} &= c_0 - \frac{0.05}{2} \left[\left(\frac{dc}{dx} \right)_m + \left(\frac{dc}{dx} \right)_{m-1} \right] \text{ etc.} \end{aligned} \quad \dots\dots(3),$$

If the values for $\frac{dc}{dx}$ vary greatly with x the following calculations have generally been used:

$$c_{m+1} = c_0 + \frac{0.01}{2} \left[\left(\frac{dc}{dx} \right)_m + 2 \left(\frac{dc}{dx} \right)_{m+1/2} + 2 \left(\frac{dc}{dx} \right)_{m+2/5} + \dots + \left(\frac{dc}{dx} \right)_{m+1} \right] \text{ etc. } \dots (4).$$

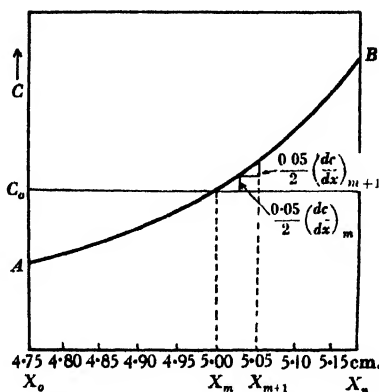


Fig. 1.

When all the values for c are calculated and plotted, the value of $\int_{x_0}^{x_n} c_i dx$ is determined. If its value is equal to $\int_{x_0}^{x_n} c_0 dx$, then the calculated values for c give the actual values in the cell. If the two integrals are not equal then all the c values should be changed by the same amount Δc , where

$$\Delta c = \frac{\int_{x_0}^{x_n} c_0 dx - \int_{x_0}^{x_n} c_i dx}{x_n - x_0} \dots (5).$$

If the cell is not rectangular in shape but sectorial, then the following expression must be fulfilled:

$$\int_{x_0}^{x_n} c_0 \frac{r_i}{r_0} dx = \int_{x_0}^{x_n} c_i \frac{r_i}{r_0} dx \dots (6).$$

If these two integrals are not equal all the calculated values of c should be changed by the amount Δc and

$$\Delta c = \frac{\int_{x_0}^{x_n} c_0 \frac{r_i}{r_0} dx - \int_{x_0}^{x_n} c_i \frac{r_i}{r_0} dx}{\frac{1}{2} \left(1 + \frac{r_n}{r_0} \right) (x_n - x_0)} \dots (7).$$

If the cell has another shape then the expression becomes more generally

$$\int_{x_0}^{x_n} c_0 f(x) dx = \int_{x_0}^{x_n} c_i f(x) dx \dots (8),$$

where $f(x)$ gives the geometrical shape of the cell, and

$$\Delta c = \frac{\int_{x_0}^{x_n} c_0 f(x) dx - \int_{x_0}^{x_n} c_i f(x) dx}{\int_{x_0}^{x_n} f(x) dx} \dots (9).$$

The calculation of the (c, x) curve from the $\left(\frac{dc}{dx}, x \right)$ curve could be made in other ways than described here, but other methods generally require two successive integrations instead of the one necessary in the method just described.

If the value for α or for some reason that for c_0 is difficult to obtain it is still possible to use the method just described if a refractive index diffusion experiment or sedimentation velocity experiment is carried out on the same solution used for the sedimentation equilibrium experiment. From either diffusion or sedimentation curve one can calculate the value for the difference $(n_1 - n_0)$ in refractive index due to the sedimenting substance.¹ It is only necessary to substitute $(n_1 - n_0)$ for c_0 , $(n_1 - n_0)$ for c , and $\frac{dn}{dx} = \frac{Z}{gab}$ for $\left(\frac{dc}{dx}\right)$ to get the $(n_1 - n_0)$, x curve.

When the (c, x) or $(n_1 - n_0)$, x curve is found, it may be used in the same way as in the absorption method to calculate the molecular weight from the formula

$$M = \frac{2RT \ln \frac{c_2}{c_1}}{(1 - V\rho) \omega^2 (x_2^2 - x_1^2)} \text{ or } M = \frac{2RT \ln \frac{n_2 - n_0}{n_1 - n_0}}{(1 - V\rho) \omega^2 (x_2^2 - x_1^2)} \quad \dots (10)$$

or we may use the differential formula from which the above integrated expression was originally derived:

$$M = \frac{RT \frac{dc}{c}}{(1 - V\rho) \omega^2 x} = \frac{RT \frac{dn}{dn_1 - n_0}}{(1 - V\rho) \omega^2 x} \quad \dots (11)^2 \quad \text{or} \quad M = \frac{RT \frac{dn}{dx}}{(1 - V\rho) \omega^2 (n_1 - n_0)} \quad \dots (12).$$

or

$$M = \frac{RT Z_1}{(1 - V\rho) \omega^2 x_1 c_1 gab} \quad \dots (13)^2 \quad \text{or} \quad M = \frac{RT Z_1}{(1 - V\rho) \omega^2 x_1 (n_1 - n_0) gab} \quad \dots (14).$$

If the solute is monodisperse and obeys the gas laws, it is generally easier to use another formula for the calculation of M , since it has been shown by Lamm [1929] (see also Pedersen [1934]) that $\frac{Z_2 x_1}{Z_1 x_2}$ could be substituted for $\frac{c_2}{c_1}$ and the expression for the molecular weight becomes accordingly

$$M = \frac{2RT \ln \frac{Z_2 x_1}{Z_1 x_2}}{(1 - V\rho) \omega^2 (x_2^2 - x_1^2)} \quad \dots (15).$$

If the solute is polydisperse, then $\frac{Z_2 x_1}{Z_1 x_2}$ differs from $\frac{c_2}{c_1}$ and the equation (15) does not give the mol. wt.; but Lansing and Kraemer [1935] have shown that equation (15) gives M_{zx} , which is of some value for finding out the degree of

¹ It may be of interest to have both the value for the area of the diffusion curve and for the area of the sedimentation curve. If the values of the two areas are the same, then the diffusing substances are equal to the sedimenting ones; if they are different, then either the solution contains some coarse disperse substances which sediment to the bottom of the cell before the measurements are started or the solution contains a low-molecular substance that does not give rise to a measurable refractive index gradient in the cell. In such a case it is not justifiable to use the area from the diffusion curve (or the analytical concentration), since in this case the refractive index gradient in the equilibrium cell will be determined only by the amount of substance corresponding to the area of the sedimentation curve (from the velocity experiment). In some experiments of the author with serum albumin (results not published) it was found that there was a strong drift in the calculated values for M as long as the analytical values for the concentrations were used; if the values calculated from the sedimentation velocity diagram were used, the drift in the molecular weight became rather small and M_{π} was not much different from M_D (the value calculated from diffusion and sedimentation constants).

² These formulae have been given before [Heidelberger and Pedersen, 1935] and their use was first mentioned by Pedersen [1935].

polydispersity. According to these authors one can calculate three different average molecular weights from the ultracentrifuge data, *viz.*:

$$\text{number-average } M_n = \frac{\sum n_i M_i}{\sum n_i} = \sum \frac{w_i}{M_i} \quad \text{.....(16)}$$

$$\text{weight-average } M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} = \frac{\sum w_i M_i}{\sum w_i} \quad \text{.....(17)}$$

$$z\text{-average } M_z = \frac{\sum n_i M_i^3}{\sum n_i M_i^2} = \frac{\sum w_i M_i^2}{\sum w_i M_i} \quad \text{.....(18),}$$

where n_i is the number of molecules of weight M_i , while w_i is the total weight of that molecular species. The usual physico-chemical methods (*e.g.* osmotic pressure measurements) generally give M_n values, whilst M_w and M_z may be obtained from ultracentrifugal data. For a monodisperse solute we have $M_n = M_w = M_z$, but for a polydisperse solute we have $M_n < M_w < M_z$, at least in very dilute solutions where the gas laws are valid.

There is often a small drift in the M_{wz} values; sometimes the values increase towards the bottom of the cell (which would indicate that the substance was heterogeneous) and sometimes they decrease towards the bottom (which ordinarily means departure from the gas laws). But since the drift is very small and sometimes in one direction and sometimes in another, it is assumed that the drift is caused by small experimental errors in the Z values or in computing the c values from the Z values.

The M_{zx} values often fluctuate widely, but the M_w and M_z values are not so very different. By a simple comparison of the determining factors in the two cases we get

$$\frac{c_2}{c_1} = \frac{z_2}{z_1} \frac{r_1}{x_2} = \frac{z_1}{z_2} \left(1 + \frac{z_2 - z_1}{z_1} \right) \quad \text{for } M_{zx},$$

$$\text{and} \quad \frac{c_2}{c_1} = \frac{c_1 + \Delta c}{c_1} = 1 + k \frac{z_1 + z_2}{2c_1} \quad \text{for } M_{wx}.$$

From this it is quite evident that the error in $\frac{z_2}{z_1}$ must be much larger than the error in $\frac{z_1 + z_2}{2c_1}$, especially when Δx is very small.

PRELIMINARY ULTRACENTRIFUGAL STUDY OF THE MILK PROTEINS.

Material. All the experiments here described were carried out on cow's milk. The fresh milk was drawn into Jena flasks and immediately brought to the laboratory, where the bulk of the butter fat was removed by skimming the milk in one of the ordinary laboratory centrifuges for a short time, after which the skim milk was spun in a closed separator bowl at high speed for about 10 min. By this procedure some fat went to the central part of the bowl and some "slime" to the periphery; these two fractions were generally thrown away and the solution, the milk plasma, was used for the experiments. Toluene was added as preservative to all solutions that were dialysed.

The first experiments were carried out on milk serum which was spun directly in the ultracentrifuge, since the large caseinogen particles sedimented to the bottom of the centrifuge-cell before the centrifuge had reached the speed necessary for the measurements on the milk serum. In two successive experiments 1.6 ml. of milk plasma were placed in the largest ultracentrifuge-cells and submitted to the action of a centrifugal field of about 100,000 times gravity for some minutes. By this procedure about 3 ml. of milk serum were obtained, an amount sufficient for carrying out four different experiments (M 1- M 4). As found by Svedberg and Fåhræus [Svedberg, 1930] and by Nichols *et al.*

[1931] it was evident that the suspended caseinogen (or calcium caseinogenate) was present in a quite polydisperse condition; at the start the solution was quite non-transparent, but as the speed increased the solution became more and more transparent and finally water-clear. By following the caseinogen separation by means of the scale method it was not possible to detect any measurable sedimentation of monodisperse substances during these milk plasma experiments.

Exp. M 1. A portion of the milk serum was investigated in a centrifugal field of about 230,000 times gravity by means of the scale method. The reference scale was made with the ultrafiltrate from the milk. The sedimentation diagram showed peaks corresponding to the molecules $\alpha + \beta$ and γ (for nomenclature see later); besides these there were probably present to a very small extent a few more species. γ was present only in a very low concentration (less than 10% of $\alpha + \beta$) and was rather difficult to identify owing to the refractive index gradient caused by the sedimentation of the large amount of lactose. During the experiment an attempt was made to take some light absorption pictures using chlorine and bromine light filters, but even with exposure times as long as 10 min. it was impossible to get any blackening on the photographic plate (the usual exposure time is 30 sec.); the thickness of the cell was 12 mm. The large light absorption is due to lactose and lactoflavin and perhaps to some other low molecular substances.

Exp. M 2. A second portion of the milk serum was dialysed in the ice-box against frequently changed buffer solution (0.06 *M* NaCl + 0.023 *M* KH_2PO_4 + 0.017 *M* Na_2HPO_4 , p_{H} about 6.7). When the lactose had disappeared the solution was investigated in the centrifuge. (In all the following experiments the reference scale was made with the corresponding buffer in the cell.) The sedimentation diagram showed the same peaks as in M 1, that is $\alpha + \beta$ and γ plus some less distinct peaks. Light absorption pictures were easily taken during the experiment and showed a fairly "uniform" curve (due to $\alpha + \beta$), when γ had sedimented down to the bottom of the cell. The sedimentation constants were for β $s_{20} = 3.2 \cdot 10^{-13}$ and for γ $s_{20} = 7.1 \cdot 10^{-13}$; the constant for α could not be determined; its presence was just evident from the curve.

Exp. M 3. A third portion of the milk serum (about 1.5 ml.) was dialysed until it was in equilibrium against a very frequently changed acetate buffer (0.2 *M* NaCl + 0.075 *M* sodium acetate + 0.025 *M* acetic acid; p_{H} 5.0). The sedimentation diagram showed the same peaks as in the preceding experiments. For β $s_{20} = 3.04 \cdot 10^{-13}$ and for γ $s_{20} = 7.4 \cdot 10^{-13}$. In this experiment it was just possible to get an estimate of the sedimentation constant for α ($s_{20} = 1.8 \cdot 10^{-13}$).

Exp. M 4. A fourth portion of the milk serum was used as in M 3, but the light absorption method was employed. On the microphotometer record the presence of α could hardly be detected, so that values calculated as the sedimentation constant for β are rather a mean of the sedimentation constants for α and β , namely $s_{20} = 2.7 \cdot 10^{-13}$. The presence of γ was quite evident from the curve, but it was not possible to calculate its sedimentation constant.

Exps. M 5, 13, 16 and 17. In these experiments the milk plasmata from two different samples of milk were dialysed for about a week in the cold (3–5°) against frequently changed phosphate buffers (in M 5: 0.06 *M* NaCl + 0.017 *M* KH_2PO_4 + 0.023 *M* Na_2HPO_4 , in the others: 0.2 *M* NaCl + 0.02 *M* KH_2PO_4 + 0.03 *M* Na_2HPO_4 , p_{H} 6.8). During this time most of the suspended caseinogen went into solution and the appearance changed from milky to opalescent. When the solution was spun in the centrifuge, it became clear at a low speed and the amount of precipitate was very small. The sedimentation diagrams from these four experiments were on the whole quite similar but entirely different from the diagrams in M 1–4. The peaks belonging to the molecules α , β and γ were likewise present here, but there were at least 7 more peaks apparent (see Fig. 2 a–d). For the 4 slowest sedimenting peaks the mean sedimentation constants were: α , 1.8; β , 3.18; γ , 6.8 and δ , $10.4 \cdot 10^{-13}$. For the other molecules the sedimentation velocity was too high to allow an accurate determination of the sedimentation constant; it ranged from 13 – $30 \cdot 10^{-13}$ (in order to get a proper determination of these high sedimentation constants the centrifugal field should be less than 100,000 times gravity). The total protein concentration in Exps. M 16 and M 17 was 1.4%.

The concentrations of α and β were not the same in the different experiments; in M 5 the concentration of α was about 1/5 of that for β , while in M 16 and

M 17 it was about twice that of β . Whether this difference in the concentration was due to differences in the two samples of milk or to different permeability of the cellophane membranes to the α molecules cannot be decided at present.

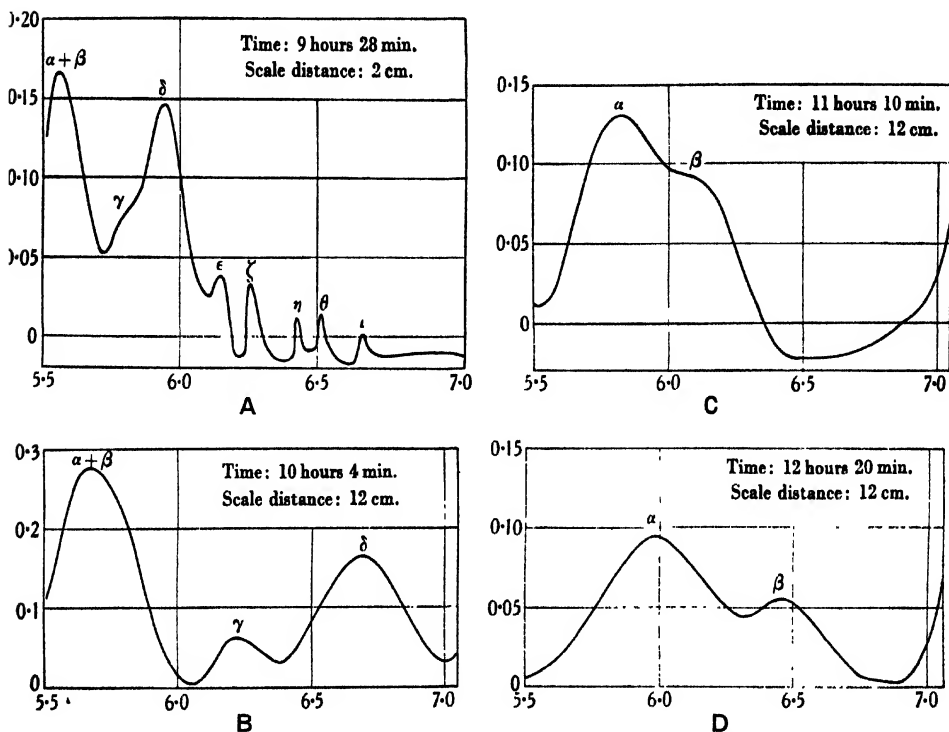


Fig. 2 A-D. *Ultracentrifugal sedimentation diagrams from M 16 obtained by the refraction method.* The abscissae (X in cm.) represent distances from the centre of rotation and the ordinates (Z in mm.) represent the scale line displacements which are proportional to the concentration gradient. Each peak on the diagram corresponds to the sedimentation of molecules of a certain size. The faster the peak moves toward the right side of the diagram, the larger the molecule; the area below the peak is proportional to the concentration of the molecule which gives rise to the peak. Exp. M 16 was carried out with a dialysed (in phosphate buffer) sample of skim milk and the solution contained most of the proteins present in milk. Centrifugal force: 260,000 times gravity. Cell thickness: 12 mm. A. Exposure taken 13 min. after reaching full speed. B. Exposure taken 49 min. after reaching full speed. C. Exposure taken 115 min. after reaching full speed. D. Exposure taken 185 min. after reaching full speed.

In the description of the experiments the different peaks of the sedimentation diagrams were named by Greek letters in such a way that the one with the smallest sedimentation constant was called α , the next smallest β and so on.

The new milk protein isolated by Kekwick has in the purified condition a sedimentation constant of about $2 \cdot 10^{-13}$, a value very close to that of the component α , so we may assume that α is the Kekwick protein.

In the same way the sedimentation constant found for β agrees very well with that found for Palmer's lactoglobulin (see Pedersen [1936]). Probably the so-called lactalbumin is a mixture of the two molecules α and β and the molecule usually referred to as lactoglobulin is γ , but this has so far not been proved.

Since all peaks from δ on appeared when the caseinogen was dissolved, we must suppose that at least some of these peaks originate from the caseinogen. Owing to its size the bulk of the caseinogen must be present in the δ peak and its sedimentation constant therefore must be $10.4 \cdot 10^{-13}$.

Some experiments were carried out to follow a fractionation process. Equal amounts of neutral saturated ammonium sulphate solution and milk plasma were mixed together. The caseinogen precipitate was centrifuged off and suspended in a solution of $0.2 M$ $\text{NaCl} + 0.02 M$ $\text{KH}_2\text{PO}_4 + 0.03 M$ Na_2HPO_4 and dialysed against the same buffer (frequently changed). The filtrate from the ammonium sulphate precipitation was dialysed under pressure against a solution of $0.06 M$ $\text{NaCl} + 0.017 M$ $\text{KH}_2\text{PO}_4 + 0.023 M$ Na_2HPO_4 until it was free from sulphate. The volume was reduced to 1.2 times the volume of milk plasma (Exp. M 6). Later the dialysis was continued against a solution of $0.2 M$ $\text{NaCl} + 0.02 M$ $\text{KH}_2\text{PO}_4 + 0.03 M$ Na_2HPO_4 until the volume was reduced to 0.4 times the volume of milk plasma (Exp. M 8).

Exp. M 6. The experiment was carried out on the filtrate from the ammonium sulphate precipitation. The bulk of the material belonged to the molecules α and β , giving sedimentation constants of 1.9 and $2.6 \cdot 10^{-13}$ respectively (an extraordinarily low value for β). Very small amounts of faster-sedimenting molecules could be observed.

Exp. M 8. The same solution was used as in M 6, but concentrated by dialysis. The concentration was 3 times that in M 6. α and β molecules were present, but the amount of α was considerably decreased; s could only be measured for β and was $3.03 \cdot 10^{-13}$. There were indications of molecules with higher sedimentation constants.

Exp. M 7. This experiment was on the precipitate from the ammonium sulphate precipitation. The centrifugal field was in this case only 180,000 times gravity. The molecules α , γ and δ were present. For δ $s_{20} = 11.1 \cdot 10^{-13}$ and for γ $6.2 \cdot 10^{-13}$, s could not be determined accurately for α owing to the low speed and the short time of the experiment. The calculated value was $1.1 \cdot 10^{-13}$, an extremely low value for α . The δ peak predominated.

The rest of the solution from M 7 was dialysed against a frequently changed solution containing $0.1 M$ sodium acetate + $0.1 M$ acetic acid. The heavy precipitate was centrifuged off, washed several times with water, and suspended in a solution containing $0.2 M$ $\text{NaCl} + 0.02 M$ $\text{KH}_2\text{PO}_4 + 0.03 M$ Na_2HPO_4 and dialysed against the same buffer (Exp. M 9). The solution from the caseinogen precipitation was dialysed under pressure against a solution containing $0.2 M$ $\text{NaCl} + 0.075 M$ sodium acetate + $0.025 M$ acetic acid (exp. M 11).

Exp. M 9. The caseinogen fraction. The concentration of the caseinogen was 1.2% . The sedimentation diagram showed the movement of just one single peak with a sedimentation constant of $11.6 \cdot 10^{-13}$. The peak was not quite symmetrical but was somewhat broader towards the meniscus, perhaps indicating some dissociation of the molecule. At the top of the cell there was an indication of a small peak sedimenting very slowly, but the concentration was too small to allow determination of the sedimentation velocity. The solution was also investigated electrophoretically by means of the scale method. It appeared quite heterogeneous with regard to electrophoretic migration showing several peaks with different migrations.

Exp. M 10. For this experiment an aliquot of the solution used in M 9 was boiled for a few minutes in order to study the influence of boiling on the sedimentation constant. The sedimentation diagram had exactly the same appearance as the one from the preceding experiment, but the sedimentation constant showed a slight increase ($s_{20} = 12.8 \cdot 10^{-13}$).

Exp. M 11. The filtrate from the caseinogen precipitation. The concentration of the proteins in this solution was considerably increased by means of pressure dialysis. The sedimentation diagrams showed the presence of α , γ and some faster-sedimenting molecules in very low concentration. For the sedimentation constant the values were: for γ $s_{20} = 6.9 \cdot 10^{-13}$ and for α $s_{20} = 1.2 \cdot 10^{-13}$ (again a very low value), but this time the experimental conditions were favourable for determining s , since α was the predominant peak.

For the next experiments the skim milk was spun for 20 min. at high speed in the closed separator bowl. By this procedure a rather large amount of material collected at the periphery of the separator bowl. This "separator slime" was suspended in a solution of $0.2\text{ }M$ $\text{NaCl} + 0.02\text{ }M$ $\text{KH}_2\text{PO}_4 + 0.03\text{ }M$ Na_2HPO_4 and dialysed against the same buffer (Exp. M 12). The milk plasma was first dialysed against $0.4\text{ }M$ acetic acid + $0.07\text{ }M$ sodium acetate for a short time and then against a solution of $0.2\text{ }M$ $\text{NaCl} + 0.075\text{ }M$ sodium acetate + $0.025\text{ }M$ acetic acid. The caseinogen precipitated very well and could be easily washed with the same buffer solution; it was suspended afterwards in a solution of $0.2\text{ }M$ $\text{NaCl} + 0.02\text{ }M$ $\text{KH}_2\text{PO}_4 + 0.03\text{ }M$ Na_2HPO_4 and dialysed against the same buffer (Exps. M 14 and M 15).

Exp. M 12. The "separator slime". On dialysing the separator slime not all of the suspended material went into solution; its appearance after several days of continuous dialysis was still quite milky, even after it had been spun in an ordinary laboratory centrifuge at high speed. "Separator slime" evidently consists of what is usually called separator slime *plus* the more coarsely dispersed part of the Ca caseinogenate. The centrifugal field was 180,000 times gravity. The sedimentation diagram was of a very complex nature showing a main peak with a sedimentation constant of $13.1 \cdot 10^{-13}$ and a number of small peaks with sedimentation constants ranging from 19 to $35 \cdot 10^{-13}$ (see Fig. 3). Later exposures showed indications of a very slowly sedimenting substance ($\alpha?$). Evidently the main peak must be due to caseinogen (δ).

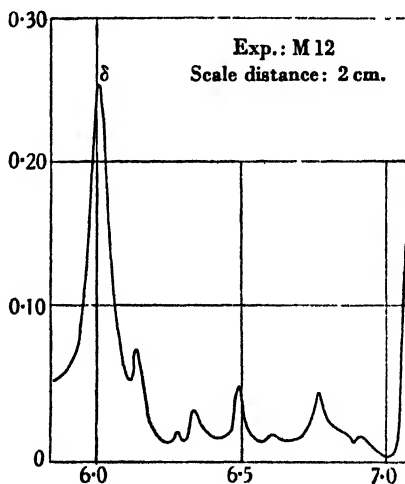


Fig. 3. Sedimentation diagram of "separator slime" + coarsely dispersed part of Ca caseinogenate dissolved in phosphate buffer p_{H} 6.8. Centrifugal force: 180,000 times gravity. Cell thickness: 12 mm. Exposure taken 20 min. after reaching full speed. Abscissa, X in cm.; ordinate, Z in mm.

Exp. M 14. The caseinogen fraction. Concentration: 2.6%. Centrifugal field: 180,000 times gravity. The sedimentation diagram showed a single peak (besides indications in the last exposures of the sedimentation of $\alpha?$) having a sedimentation constant of $23 \cdot 10^{-13}$. The peak was quite unsymmetrical being much broader toward the top part of the cell and the apparent diffusion was much higher than would be expected for a molecule with such high sedimentation constant. It must be concluded that the substance is dissociating during sedimentation and producing new molecules with smaller sedimentation constants in the part of the boundary, where the concentration is lowest. No molecules with higher sedimentation constants than $23 \cdot 10^{-13}$ could be detected in the diagram (Fig. 4).

Exp. M 15. On caseinogen from the same solution as in M 14, but with a protein concentration 1/3 of the concentration in M 14. Centrifugal field: 180,000 times gravity. The sedimentation picture from this experiment was entirely different from that of M 14 (compare Figs. 4 and 5, which were taken almost at the same time from the start of the centrifuge). In this experiment the main component showed a sedimentation constant of $11.1 \cdot 10^{-13}$, but besides this main

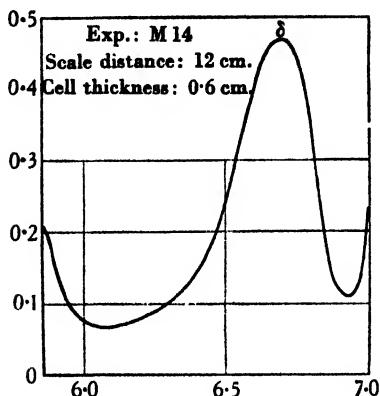


Fig. 4.

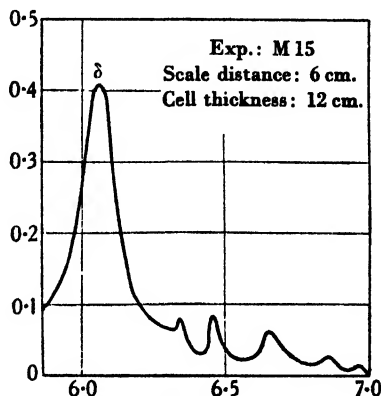


Fig. 5.

Fig. 4. Sedimentation diagram of a caseinogen fraction. Concentration: 2.6%. Centrifugal force: 180,000 times gravity. Exposure taken 24 min. after reaching full speed. Abscissa, X in cm.; ordinate, Z in mm.

Fig. 5. Sedimentation diagram of the same caseinogen fraction as in Fig. 4 after dilution. Concentration: 0.9%. Centrifugal force: 180,000 times gravity. Exposure taken 20 min. after reaching full speed. Abscissa, X in cm.; ordinate, Z in mm.

component the sedimentation diagram showed several different peaks corresponding to particles with sedimentation constants ranging from 24 to $36 \cdot 10^{-13}$. In the later exposures the peak became very broad and unsymmetrical thus indicating dissociation of the molecule.

DISCUSSION.

From the experiments just described some conclusions may be drawn. In the first experiments (M 1-4), where the suspended Ca caseinogenate was removed from the milk plasma by ultracentrifuging, it was found that practically the whole material was present as molecules α , β and γ , where α is a low-molecular protein isolated by Kekwick, β the lactoglobulin isolated by Palmer and γ corresponds probably to what is generally called lactoglobulin. It may be assumed that what was earlier called lactalbumin is a mixture consisting mainly of $\alpha + \beta$.

The results mentioned are very similar to the earlier results of Philippi. In his experiments however the caseinogen fraction was removed by isoelectric precipitation. Comparison of the two series of experiments shows that it makes little or no difference whether caseinogen is removed by isoelectric precipitation or by ultracentrifuging.

Since only the molecules α , β and γ could be found in the milk serum, we must suppose that the caseinogen is only present in the milk as very coarsely dispersed particles (Ca caseinogenate) and that the lactalbumin and lactoglobulin (α , β and γ) molecules present in the milk are small as compared with the smallest of the caseinogen particles.

The experiments carried out on the caseinogen fraction have shown that this protein is polydisperse and of a very complex nature; it was found that the

sedimentation constant (and therefore the dispersity) of the caseinogen was markedly dependent upon its concentration; s decreased with decreasing protein concentration. Svedberg *et al.* [1930, 1, 2] found in their studies on the Hammarsten and on the Van Slyke and Baker caseinogens a similar concentration effect on the sedimentation constant. The protein prepared by them according to both methods was polydisperse and the sedimentation constants found for this caseinogen were considerably lower than the values found in the present investigation. It is possible that the disagreement may be due to the different salt concentrations used, 0.017 M in the earlier work and 0.25 M in the present paper. Lundgren in Upsala found (results not yet published) that for a protein which dissociates by dilution, the dissociation is much more pronounced in dilute salt solutions than in the 0.2 M solution usually applied. In this work it has not been possible to identify in the caseinogen fractions a protein corresponding to the homogeneous one which Svedberg *et al.* isolated from the Hammarsten caseinogen by means of hot acidified 70% alcohol.

From the few experiments done in this investigation it could not be ascertained whether the peak named α corresponded to one and always the same substance or whether it corresponded to two different substances.

The work is being continued.

SUMMARY.

1. A description has been given of a modified method for calculating sedimentation equilibrium concentrations from the refractive index data by the method of Lamm.

2. In some preliminary experiments on milk plasma it has been shown that the caseinogen is present in the milk only as a coarse polydisperse suspension.

3. When the caseinogen has been brought into solution in sodium and potassium phosphate buffers it is of a very complex nature, the degree of dispersity being dependent (amongst other factors) on the caseinogen concentration.

4. The milk serum was found to contain at least three different proteins: a substance of low mol. wt. isolated by Kekwick, the lactoglobulin of Palmer and what is usually called lactoglobulin.

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CXXXIX. ULTRACENTRIFUGAL AND ELECTROPHORETIC STUDIES ON THE MILK PROTEINS.

II. THE LACTOGLOBULIN OF PALMER.

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It is generally assumed that milk serum contains two different proteins: lactalbumin and lactoglobulin. However, Palmer [1934] recently isolated a crystalline, globulin-like protein from the albumin fraction of cow's milk. Recent ultracentrifugal studies by Philippi¹ have shown the presence of at least three different proteins in milk serum: a protein of rather low molecular weight, isolated later on by Kekwick,¹ the lactoglobulin studied by Palmer [1934] and a protein with a molecular weight higher than that of the latter.

Recent investigations by the author gave similar results [Pedersen, 1936]. This paper gives the result of an ultracentrifugal and electrophoretical study of the protein isolated by Palmer.

The lactoglobulin used throughout this investigation was kindly put at the disposal of Prof. The. Svedberg by Prof. Sørensen. It was prepared by Dr Palmer at the Carlsberg Laboratory from cow's milk according to a modification of the method worked out by Dr Palmer himself. This preparation had been recrystallised four times. The material was received in Upsala as a wet crystal paste containing some toluene as preservative and was stored in this condition in the refrigerator (3–5°). A part of the lactoglobulin was later dried in a desiccator over P₂O₅ and kept in the desiccator. These two materials behaved identically in the centrifuge and in electrophoresis. At the end of this investigation a few experiments were carried out using another material prepared by R. A. Kekwick in Upsala according to the method of Palmer. The sedimentation values found for this material were identical with the earlier ones.

*The specific refraction increment and specific volume of lactoglobulin.*² The determinations of the specific refraction increment were carried out on solutions prepared by dissolving the lactoglobulin in 0.5 *M* NaCl solution and dialysing it in cellophane bags against a 0.5 *M* NaCl solution in water. The determinations were carried out by means of a differential prism apparatus arranged for photographic registration of the undeviated and refracted beams. The concentrations were determined by drying a weighed amount of protein solution and a weighed amount of dialysate to constant weight (105–110°).

In Table I are given the results of two independent determinations. A micro-Kjeldahl determination (Gunning-Pregl method) gave for total nitrogen of the first solution 15.14 % and for the second 15.26 % in good agreement with Palmer's value which was 15.3 %.

The specific volume of lactoglobulin was found to be 0.7514 at 20° which is in good agreement with the values found for other proteins.

¹ Results to be published soon.

² The author is much indebted to Mr Kjell Andersson for having carried out these determinations.

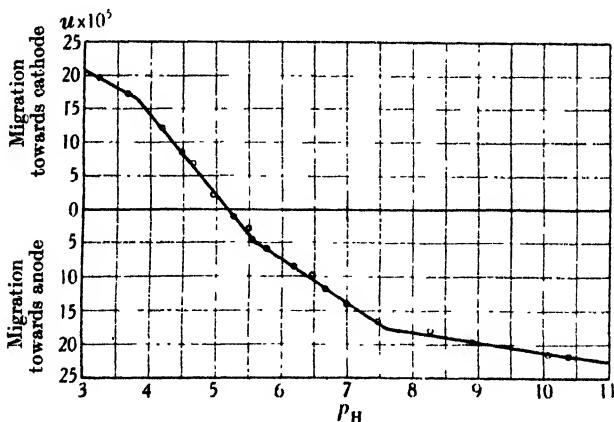
Table I. *The specific refraction increment (α) of lactoglobulin in 0.5M NaCl solution.*

Protein concentration		λ in $m\mu$			
g./100 g. sol.	g./100 ml.	366	436	546	579
1.231	1.2566	196.2	189.3	181.3	181.2
1.853	1.8934	196.4	189.2	182.3	180.9
Mean for α :		196.3	189.2	181.8	181.0

The isoelectric point and the electrophoretic mobility of the lactoglobulin. These experiments were carried out by means of a special moving boundary method worked out by Tiselius [1930] and Pedersen [1933]. In this method the protein solution is introduced into a quartz U-tube below a solution of the same buffer

Table II. *Electrophoretic mobility of lactoglobulin.*

Exp. No.	p_H	Buffer	Migration	$u \cdot 10^5$
17	3.22	Acetate	Cathodic	19.6
3	3.66	"	"	17.3
7	4.18	"	"	12.1
6	4.48	"	"	8.5
2	4.65	"	"	6.8
8	4.95	"	"	2.2
4	5.26	"	Anodic	1.1
5	5.49	"	"	2.9
9	5.55	"	"	4.6
13	5.76	Phosphate	"	5.9
10	6.18	"	"	8.5
12	6.46	"	"	9.8
19	6.66	Phosphate-borate	"	11.8
11	7.00	Phosphate	"	14.0
14	7.47	"	"	15.6
20	8.27	Phosphate borate	"	18.0
15	8.92	"	"	19.7
16	10.07	Phosphate-carbonate	"	21.5
18	10.38	"	"	21.7

Fig. 1. *Electrophoretic mobility of lactoglobulin.*

composition as that in which the protein is dissolved. The migration of the boundary is followed by taking photographs from time to time (in the short wave ultraviolet, λ below $270m\mu$). These photographs are registered later by

the microphotometer in the same way as the centrifuge plates and the movement of the boundary is determined from these records.

All the experiments were carried out at 20.0°. The protein concentration in these experiments varied between 0.15 and 0.20 g. per 100 ml. The acetate buffers had all a constant sodium acetate and a varied acetic acid concentration, viz. 0.02 *M* sodium acetate + *xM* acetic acid. The other buffers were made up to constant ionic strength $\mu = 0.02$. The mobility *u* is measured in cm.²/sec. · volt⁻¹.

In Table II and Fig. 1 are given the values found for the mobility of lactoglobulin in the different buffers. From these figures it is seen that the isoelectric point of lactoglobulin is at p_H 5.20 in acetate buffers of the composition 0.02 *M* sodium acetate + *xM* acetic acid. The slope of the mobility curve at the isoelectric point $\left(\frac{du}{dp_H}\right) = 11.9 \cdot 10^{-5}$. The curves show no sign of inhomogeneity.

The (*u*, p_H) curve is of a very characteristic shape with abrupt changes in the slopes at the following p_H values: 3.8, 5.6 and 7.7; otherwise the points lie on straight lines. The change in the slope at p_H 7.7 corresponds to the jump in the p_H stability curve (see later). The change in slope at p_H 5.6 may perhaps be explained by a change from the simple uni-univalent acetate system to the more complicated phosphate system: on the other hand there is a change in the sedimentation constant slightly above p_H 5. The change at p_H 3.8 does not seem to correspond to a change in the sedimentation constant.

Sedimentation velocity experiments. The sedimentation constant was determined between p_H 1 and 11. The buffer solutions generally were made up in such a manner that the concentration of NaCl was 5–10 times the concentration of the proper buffer substance in order to reduce both primary and secondary charge effects.¹ The protein solutions were made up immediately before starting the experiment except in some cases indicated in Table III B. Experiments 21, 34 and 35 were carried out in order to see whether it was possible to detect a secondary charge effect at these p_H values, where the protein must be very strongly charged; the experiments however did not give a very definite answer, although the values for s_{20} are of the expected orders, viz. $2.79 \cdot 10^{-13}$ for KCl, $2.92 \cdot 10^{-13}$ for NaCl and $2.98 \cdot 10^{-13}$ for LiCl. From the values given in Table III A and Fig. 2, it is seen that the sedimentation constant of lactoglobulin is divided into three different groups. The first group between p_H 1 and 5 shows a mean sedimentation constant of $2.95 \cdot 10^{-13}$ (mean of 19 values). The next group between p_H 5.2 and 7 has a mean sedimentation constant of $3.12 \cdot 10^{-13}$ (mean of 13 values). The third group, p_H 7.6–9.8, gives $s_{20} = 2.76 \cdot 10^{-13}$ (13 values). Between these three groups intermediate values are found. On the alkaline side of the third group there seems to be a rise in the sedimentation constant which increases, if the solution is allowed to stand for some time at room temperature (compare too equilibrium Exp. 13). At still higher p_H values (above 11) there is a strong fall in the sedimentation constant at the same time as the light absorption is increased to about three times its normal value indicating profound changes in the lactoglobulin molecule.

The influence of the lactoglobulin concentration on the sedimentation constant (compare Exps. 40–46) is extremely small and hardly beyond the limits

¹ The primary charge effect is caused by the potential gradient set up by the unequal "sedimentation tendency" of the protein and its "gegenionen". The secondary charge effect is caused by the influence of the potential gradient set up by the sedimentation of the unequally dense salt ions on the sedimenting charged colloidal particles. Whereas the primary charge effect is reduced by the mere addition of electrolytes, the secondary effect is reduced by the addition of electrolytes with equally dense cations and anions.

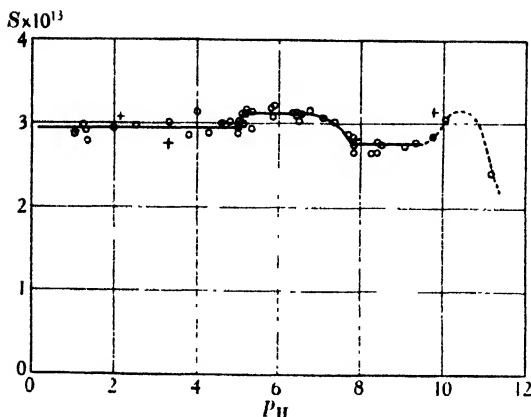
Table IIIA. *Sedimentation velocity of lactoglobulin.*

Centrifugal force 240,000–290,000 times gravity except in Exp. 7, where it was 570,000 times gravity. Thickness of column of solution: 0.2–2.2 cm. Source of light: mercury lamp. Light filter: chlorine and bromine (except in the experiments marked with *L*, where the refractometric method of Lamm was used). Plates: imperial process. Time of exposure: 20–45 sec. Metol quinol developer 1 min.

Exp. No.	Lactoglobulin concentration %	Solvent system	Total salt molarity	p _H of solution	s ₂₀ · 10 ¹³
23	0.31	NaCl, HCl	0.59	1.04	2.88
21	0.3	"	0.55	1.28	2.92
35	0.3	LiCl, HCl	0.55	1.24	2.98
34	0.3	KCl, HCl	0.55	1.33	2.79
25	0.31	NaCl, HCl	0.51	1.98	2.94
22	0.35	NaCl, citrate, HCl	0.55	2.53	2.97
12	0.30	NaCl, sodium acetate, acetic acid	0.55	3.32	3.01
48	0.31	" "	0.24	3.81	2.86
14	0.30	" "	0.15	4.00	3.14 ?
50	0.32	" "	0.53	4.28	2.88
5	0.3	" "	0.16	4.6	3.00
47	0.31	" "	0.24	4.68	2.99
27	0.3	" "	0.58	4.80	3.02
45	0.05	" "	0.275	5.00	2.99
43	0.06	" "	0.275	5.00	2.92
44	0.12	" "	0.275	5.00	2.90
42 L	0.32	" "	0.275	5.00	3.03
41 L	0.64	" "	0.275	5.00	2.99
40 L	1.28	" "	0.275	5.00	2.97
46 L	4.47	" "	0.275	5.00	(2.85)*
38	0.3	NaCl, KH ₂ PO ₄ , Na ₂ HPO ₄	0.59	5.12	3.11
39	1.04	" "	0.59	5.12	2.99
3	0.3	NaCl	0.2	About 5.2	3.12
1	0.15	"	0.2	About 5.2	3.17
49	0.31	NaCl, KH ₂ PO ₄ , Na ₂ HPO ₄	0.29	5.33	2.94
8	0.31	KH ₂ PO ₄ , Na ₂ HPO ₄	0.18	5.36	3.14
9	0.30	" "	0.16	5.81	3.19
16	0.30	NaCl, KH ₂ PO ₄ , Na ₂ HPO ₄	0.12	5.87	3.08
15 L	1.46	" "	0.12	5.87	3.21
7	0.3	" "	0.2	6.33	3.13
52	0.33	" "	0.58	6.41	3.14
53	0.34	" "	0.60	6.41	3.10
51	0.32	" "	0.58	6.5	3.03
2	0.15	" "	0.23	6.57	3.12
54	0.33	" "	0.25	6.77	3.16
4	0.3	" "	0.20	7.09	3.07
37	0.3	" "	0.54	7.36	3.02
10	0.3	KH ₂ PO ₄ , Na ₂ B ₄ O ₇	0.27	7.69	2.88
32	0.33	NaCl, KH ₂ PO ₄ , Na ₂ B ₄ O ₇	0.64	7.81	2.84
19	0.3	" "	0.64	7.81	2.66
31	0.33	" "	0.64	7.84	2.75
11	0.3	KH ₂ PO ₄ , Na ₂ B ₄ O ₇	0.25	8.25	2.65
55	0.33	NaCl, KH ₂ PO ₄ , Na ₂ B ₄ O ₇	0.53	8.38	2.66
56	0.33	" "	0.55	8.38	2.70
13	0.30	KH ₂ PO ₄ , Na ₂ B ₄ O ₇	0.28	8.4	2.78
17 L	1.22	NaCl, KH ₂ PO ₄ , Na ₂ B ₄ O ₇	0.11	8.5	2.75
28	0.3	NaCl, Na ₂ B ₄ O ₇ , Na ₂ CO ₃	0.54	9.06	2.73
36	0.3	" "	0.58	9.33	2.78
6	0.3	" "	0.19	9.74	2.85
18	0.3	" "	0.58	9.75	2.85
29	0.3	" "	0.58	10.05	3.05
20	0.1	NaCl, Na ₂ HPO ₄ , NaOH	0.53	11.16	2.42†

* Not used in averaging, owing to the high protein concentration.

† Strong change in light absorption.



values from Table III A. + values from Table III B.

Fig. 2. p_H stability diagram of lactoglobulin.

Table III B. *Sedimentation velocity of lactoglobulin.*

Conditions as in Table III A with the exception that in this case the lactoglobulin solution was made some time before the experiment started. (The solutions contained toluene as a preservative.)

Exp. No.	Protein conc. %	Solvent system	Total salt molarity	p_H of solution	$s_{20} \cdot 10^{13}$	Time interval between making up solution and experiment
24	0.31	NaCl, HCl	0.59	1.04	2.89	2 days at 20
30	0.3	NaCl, citrate, HCl	0.55	2.15	3.07	?
12 a	0.3	NaCl, sodium acetate, acetic acid	0.55	3.32	2.76	2 days at 20
33	0.3	NaCl, KH_2PO_4 , $Na_2B_4O_7$	0.64	7.84	2.92	4 days at 20
26	1.03	NaCl, $Na_2B_4O_7$, Na_2CO_3	0.58	9.75	3.15	14 days at 20

of experimental error except perhaps in the case of the 4.5% solution. In this respect lactoglobulin differs from other proteins that have so far been studied over a wider concentration range.

In the experiments marked with L the initial concentration of the sedimenting protein was calculated from the sedimentation diagrams as described by McFarlane [1935]. For these calculations the exposures were used only where the whole sedimentation peak was free from the meniscus and where it had not yet reached the bottom of the cell. In Table IV the values calculated from the single exposures are given in chronological order. It is seen that the mean values differ very little from the analytical concentration except in Exp. 46, where possibly the value given for analytical concentration is too high.

Table IV. *Concentration of lactoglobulin calculated from sedimentation diagram.*

Exp. No.	Concentration calculated from the single exposures	Mean conc. g./100 ml.	Anal conc. g. 100 ml.
15	1.50, 1.50, 1.46, 1.45, 1.59, 1.46, 1.48, 1.51	1.49	1.46
17	1.24, 1.22, 1.23, 1.22, 1.20, 1.19	1.22	1.22
40	1.23, 1.24, 1.25, 1.21	1.23	1.28
41	0.615, 0.667, 0.637	0.640	0.64
42	0.313, 0.324, 0.349, 0.329, 0.337, 0.326, 0.326, 0.319	0.328	0.32
46	4.24, 4.17, 4.39, 4.27, 4.05, 4.11	4.21	4.47*

* No exact analysis on this solution, the value 4.47 is the maximum possible value.

The sedimentation diagrams often showed a small irregularity between the meniscus and the lactoglobulin curve indicating the presence of a small amount of a substance with a lower sedimentation constant than that of lactoglobulin: the concentration of this substance was too small to give even an approximate value for its sedimentation, but in all cases it was lower than that for lactoglobulin. This substance is probably either some degradation product of lactoglobulin or more likely the low-molecular protein of Kekwick mentioned before.

Diffusion constant. The author is indebted to Mr Polson for carrying out some diffusion measurements on lactoglobulin. The experiments were carried out by means of the refractometric method described by Lamm and Polson [1936]. The values given in Table V for D_{20} are the values reduced to a water basis and infinite protein dilution.

Table V. *Diffusion of lactoglobulin.*

(Determinations by A. G. Polson.)						
Exp. No.	Protein conc. %	Solvent	p_{11}	$D_{20} \cdot 10^7$	Mean $s_{20} \cdot 10^{13}$ at same p_{11}	M_D
1	1.0	0.2 <i>M</i> NaCl	5.0	7.27	2.95	39,700
		0.038 <i>M</i> sodium acetate				
		0.012 <i>M</i> acetic acid				
2	1.0	0.001 <i>M</i> Na ₂ HPO ₄	5.4	7.19	3.12	41,600
		0.019 <i>M</i> KH ₂ PO ₄				
		0.2 <i>M</i> NaCl				
3	1.0	0.0375 <i>M</i> KH ₂ PO ₄	6.1	7.32	3.12	42,400
		0.0125 <i>M</i> Na ₂ HPO ₄				
		0.2 <i>M</i> NaCl				
4	1.0	0.02 <i>M</i> Na ₂ CO ₃	9.3	6.88	2.76	39,200
		0.02 <i>M</i> Na ₂ B ₄ O ₇				
		0.2 <i>M</i> NaCl				
Mean value						40,700

According to Svedberg [1925; 1927] it is possible to calculate the mol. wt. of a solute from its sedimentation and diffusion constants, if one assumes that the frictional coefficient is the same for a sedimenting as for a diffusing particle. Then we have

$$M_D = \frac{RT \cdot s}{D(1 - v\rho)}$$

In the deduction of this formula no assumptions have been made about the shape of the particle; it is therefore of general use.

In column 7 of Table V are given the values calculated from this formula.

Sedimentation equilibrium experiments. The ultracentrifuge technique by means of sedimentation equilibrium measurements allows a direct determination of the mol. wt. [Svedberg, 1925; 1926]. 17 equilibrium experiments were carried out with different concentrations of lactoglobulin and different buffers. The speed was varied between 8700 and 12500 r.p.m. The temperature was in all cases 20°. Equilibrium was generally obtained after 5-7 days.

The measurements of the concentration gradient in the centrifuge cell were made by means of the refractometric method of Lamm [1928; 1929; 1933] and the calculations of the mol. wt. were carried out in the two different ways described earlier [Pedersen, 1936]. Only in the case of a monodisperse system and where the gas laws are valid can it be expected that the two methods should give the same result, *viz.* the correct mol. wt. All the average mol. wts. were calculated according to the method of Lansing and Kraemer [1935] as described in the preceding paper [Pedersen, 1936].

In Tables VI and VII are given the more detailed data for equilibrium in Exp. 5 at p_H 6.36 where the lactoglobulin is monodisperse, and Exp. 13 at p_H 9.75 where it is polydisperse.

Table VI. *Lactoglobulin equilibrium, Exp. 5.*

Concentration of lactoglobulin at start: 0.1625 g./100 ml. Buffer solution: 0.5 *M* NaCl + 0.0625 *M* KH_2PO_4 + 0.0625 *M* Na_2HPO_4 . p_H = 6.36. Thickness of cell: 1.201 cm. Bottom of cell: 5.17 cm. from axis of rotation. Meniscus of solution: 4.83 cm. from axis of rotation. Optical scale distance: 8.77 cm. G = 1.015. Light filter: Corning nickel oxide (λ = 366 $m\mu$). α_{900} = $196.3 \cdot 10^{-5}$. Temperature: 20.0°. Rev. per sec.: 182.8. Γ = 0.7514. ρ = 1.034. $(1 - \Gamma\rho)$ = 0.2231. Exposures used taken 119, 125, 144, 149 and 167 hours from the start. Cell not sector-shaped but with

$$f(x) = -2.016x + 11.483 \quad (\text{Eq. 8 and 9 Pedersen [1936]}).$$

x	z in μ	$\frac{dc}{dx}$	c	M_{wx}	M_{zx}
4.87	57.5	—	—	—	—
4.90	62	0.295	0.130	38,300	39,600
4.95	70.5	0.336	0.146	38,400	32,300
5.00	78.5	0.374	0.164	37,800	32,600
5.05	87.5	0.417	0.184	37,200	37,000
5.10	99	0.471	0.206	37,200	39,500
5.15	113	0.538	0.231	37,500	—
				$M_w = 37,600$	Mean 36,200*

* Too strong fluctuation in the M_{zx} to allow an accurate determination of M_z .

Table VII. *Lactoglobulin equilibrium Exp. 13.*

Concentration of lactoglobulin at start: 0.341 g./100 ml. Buffer solution: 0.5 *M* NaCl + 0.0208 *M* $\text{Na}_2\text{B}_4\text{O}_7$ + 0.0625 *M* Na_2CO_3 . p_H = 9.75. Thickness of cell: 0.999 cm. Bottom of cell: 5.18 cm. from axis of rotation. Meniscus of solution: 4.75 cm. from axis of rotation. Optical distance: 8.79 cm. G = 1.011. Light filter: Corning nickel oxide (λ = 366 $m\mu$). α_{900} = $196.3 \cdot 10^{-5}$. Temperature: 20.0°. Rev. per sec.: 180.0. Γ = 0.7514. ρ = 1.032. $(1 - \Gamma\rho)$ = 0.2247. Exposures used in calculations taken 187, 209, 215, 230, 239 and 256 hours from start. Cell not sector-shaped but with parallel walls.

x	z in μ	$\frac{dc}{dx}$	c	M_{wx}	M_{zx}
4.80	77	0.442	0.209	37,400	62,600
4.85	93	0.534	0.234	39,900	61,000
4.90	112	0.643	0.263	42,300	65,900
4.95	137	0.786	0.299	45,000	70,000
5.00	170	0.975	0.343	48,200	75,800
5.05	215	1.234	0.397	52,200	83,800
5.10	279	1.601	0.468	56,800	87,500
5.15	367	2.106	0.560	61,800	—
				$M_w = 50,200$	$M_z = 77,800$

The drift in the values in Exp. 5 is within limits of experimental errors [Pedersen, 1936] whereas the drift in Exp. 13 certainly is real and means that the molecules at this p_H are aggregating. It is characteristic that the equilibrium in Exp. 13 took a much longer time to be established than usual, probably because the aggregation process is taking place slowly which is in agreement with the two velocity Exps. 18 and 26. The first of these was done immediately after mixing a portion of the same solution as the one used in equilibrium Exp. 13; the sedimentation constant was $2.85 \cdot 10^{-13}$ which is a little higher than the mean value for that p_H . Exp. 26 was done on the stock solution used to prepare the equilibrium solution; this had been standing at 20° for a fortnight, and the sedimentation constant was $3.15 \cdot 10^{-13}$, which is much higher than the mean value for that p_H , likewise indicating aggregation.

Table VIII gives a summary of all the equilibrium experiments. It can be seen that the most probable mol. wt. found from sedimentation equilibrium is about 38,000. There is some discrepancy between this value and the values

Table VIII. *Sedimentation equilibrium experiments on lactoglobulin.*

Exp. No.	Initial protein conc. %	Buffer solution	Total buffer conc. M	p_H	Rev. per sec.	M_w	M_z
1	0.21	NaCl, KH_2PO_4 , Na_2HPO_4	0.24	5.4	180	37,700	38,500
2	0.42	"	0.24	5.4	180	37,300	38,600
3	0.21	"	0.24	5.4	204	35,300	35,700
4	0.42	"	0.24	5.4	204	36,400	36,600
5	0.16	"	0.63	6.4	183	37,600	(36,200)*
6	0.16	"	0.63	6.4	208	36,000	(36,300)*
7	0.30	"	0.12	5.9	145	37,900	39,800
8	1.47	"	0.12	5.9	145	40,700	42,500
9	0.30	"	0.12	5.9	180	40,800	37,800
10	1.47	"	0.12	5.9	180	41,300	40,500
11†	1.47	"	0.12	5.9	145	37,500	—†
12†	1.47	"	0.12	5.9	180	36,700	—†
13	0.34	NaCl, $Na_2B_4O_7$, Na_2CO_3	0.58	9.8	180	50,200	77,800
14	0.41	NaCl, KH_2PO_4 , $Na_2B_4O_7$	0.64	7.8	180	35,500	40,900
15	0.84	"	0.64	7.8	180	36,400	(40,500)*
16	0.41	"	0.64	7.8	204	37,100	39,900
17	0.84	"	0.64	7.8	204	36,300	41,000
Average for No. 1-12						37,900	38,800
" 14-17						36,300	40,600

* Ordinary mean values due to strong fluctuation in M_{zx} values.

† The new refractometric method of Lamm [1934] used.

‡ Too strong fluctuations in M_{zx} to allow any calculations of average values.

calculated from the diffusion and sedimentation constants, although some of the single values for M_w and M_z agree with the M_D values. If we consider all the values for M_w , M_z and M_D we get as probable mol. wt. for lactoglobulin 39,000.

DISCUSSION.

This investigation shows that the lactoglobulin prepared according to the method of Palmer [1934] from the lactalbumin fraction of cow's milk is a monodisperse protein with a mol. wt. of 39,000 and an isoelectric point of 5.19 (in acetate buffers). From the result of the sedimentation equilibrium experiments and from the mol. wts. calculated from sedimentation and diffusion constants we must assume that the mol. wt. of lactoglobulin is independent of the hydrogen ion concentration of the solution from the most acid values studied up to p_H 9. At still higher p_H values the lactoglobulin undergoes a slow aggregation as found from sedimentation equilibrium and velocity experiments. Above p_H 11 the light absorption increases strongly and the sedimentation constant decreases, indicating profound changes in the molecule (probably hydrolysis).

If we assume that the mol. wt. is constant between p_H 1 and 9 and take the small variations in M_D as being due to experimental errors, then we have to explain the change in the sedimentation constant at p_H around 5 and 7.5. For an unchanged mol. wt. change of the sedimentation constant must be due to change of the molecular frictional constant. A change in the molecular frictional constant can be due to two causes: either the shape or the kinetic volume of the particle has been altered, *e.g.* due to change in hydration. From sedimentation

and diffusion data alone it is impossible to distinguish between these two modes of change. The electrophoretic mobility (u , p_H) curve shows a characteristic jump at about the same p_H values as the sedimentation constant. A mere change in the shape of the particles would probably not explain the change in slope in the (u , p_H) curve, since it has often been found (for instance in Abramson's work with protein-covered quartz particles) that the size and shape of the particle influence the electrophoretic mobility very little. The jump in the (u , p_H) curve is therefore probably due to some change in the ionisation of the molecule.

The fact that the sedimentation constant hardly changes with concentration shows that the dissociation tendency of lactoglobulin is negligible.

From the constancy of the mol. wt. one cannot infer that the lactoglobulin molecule remains unchanged in such a manner that it should still possess its original properties, when it is brought back to a p_H around the isoelectric point after having been exposed to acid or alkali. Two electrophoresis experiments have shown that changes in the lactoglobulin molecule take place under these conditions. In one of these experiments some lactoglobulin was dissolved in dilute HCl to give a p_H of about 1.8 and was left in this solution for 1 day; after that time the solution was first dialysed against distilled water and later against phosphate buffer at p_H 6.15. The dialysate was changed several times. The electrophoretic mobility was determined and gave $u = 9.05 \cdot 10^{-5}$, whereas the normal value is $8.3 \cdot 10^{-5}$, so that it may be assumed that the lactoglobulin has probably undergone some change. Another sample of lactoglobulin was first treated at p_H 10 for 1 day and then dialysed against water and phosphate buffer (p_H 6.15). While the HCl-treated lactoglobulin gave uniform curves but a somewhat different mobility from the normal, this lactoglobulin gave quite heterogeneous curves showing that more particles were present with different mobilities. This indicates that profound changes in the molecule have occurred at p_H 10.

SUMMARY.

1. An ultracentrifugal study has been carried out on the lactoglobulin prepared according to Palmer.
2. The protein was found to be homogeneous and to have a mol. wt. of about 39,000.
3. The mol. wt. was found to be constant between p_H 1 and 9 but the sedimentation constant varied indicating a change in the molecular frictional constant at about p_H 5 and 7.5.
4. An electrophoretic study of lactoglobulin has shown that its isoelectric point is at p_H 5.19 in acetate buffers.

The author wishes to express his gratitude to Prof. Svedberg for his inspiring interest in this work.

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CXL. CONSTITUTION OF CERTAIN NUTSHELLS.

I. THE SEED-COAT OF *BERTOLLETIA EXCELSA* (BRAZIL-NUT).

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SURVEY of the literature reveals that little or no work has been done on the constitution of nutshells. In fact a quantitative determination of the main constituents of peanut hull (*Arachis hypogea*) is the only investigation recorded for this type of tissue [Emley, 1928]. The following table gives the results of Emley's analysis:

	%
Water-soluble, cold	8.5
Water-soluble, hot	9.8
1% alkali-soluble, cold	21.5
Ether-soluble	0.5
Alcohol-soluble	3.2
Pentosans	18.5
Lignin	33.4
Cellulose	44.9
Pentosans in pulp	13.5

One point stands out clearly, that the pentosan content of the shell is high and in particular that part of the pentosan associated with the cellulose is large compared with that from the celluloses of other lignified tissues.

These figures suggested that other "nut" shells might repay investigation by bringing to light general characteristics of this group of tissues. The results obtained from the first of these investigations, on the Brazil-nutshell, are recorded in this paper.

Preliminary analysis.

The shells carefully freed from all traces of the oily endosperm were ground to a fine powder. Very fine grinding was essential in order that the cellulose might be obtained from the lignocellulose complex without considerable oxidation taking place. No attempt was made to separate the layers which made up the shell. The following table gives an average of the analyses of the samples taken from the bulk:

	%
Benzene-soluble	4.69
Alcohol-soluble	1.06
Water-soluble	1.64
5% NaOH (cold)-soluble	16.00
Lignin	53.25
Cellulose	25.47
	<hr/> 102.11

Preparation of fractions.

The mass of the powdered shell was treated by the methods used in the quantitative analysis. The benzene, alcohol and water extracts were prepared with an extractor working on the Soxhlet principle and holding about 1000 g.

at each filling. These three fractions were obtained by evaporation of the solvents under reduced pressure. The NaOH-soluble fraction was obtained by treating the residue from the water extraction with 5% NaOH in the proportion of 100 g. of the residue to 1 l. of solution for 24 hours at room temperature with constant stirring. The residue was filtered off and the filtrate acidified with a slight excess of acetic acid. The bulky, fawn-coloured precipitate was centrifuged. This precipitate designated hemicellulose A was washed on the centrifuge with increasing concentrations of alcohol, finally with absolute alcohol and ether and dried in a vacuum desiccator. When the filtrate was neutralised with solid sodium carbonate and gently warmed a second precipitate came down. This precipitate, hemicellulose A 1, was separated, washed on the centrifuge and dried as before. The filtrate was dialysed for 8 days: it was then concentrated *in vacuo* to low bulk, filtered to remove a small amount of precipitate and treated with 3 vols. of 95% alcohol. The resulting precipitate was collected, washed and dried as before—hemicellulose B. The lignin was prepared by treating part of the NaOH-insoluble residue with fuming HCl overnight, diluting to 5% and boiling for 6 hours. The residue, washed free from acid and dried by treatment with alcohols of increasing strength and with ether, was regarded as lignin. Cellulose was prepared by chlorination of part of the NaOH-insoluble residue and solution of the chlorolignin in either 3% sodium sulphite or 95% alcohol. Both methods gave similar results.

Benzene-soluble fraction.

After removal of the solvent the residue was obtained as a dark brown viscous mass which on standing deposited a white solid "stearin".

The crude product had the following characteristics:

Sap. equiv.	199.6
I.V.	110.9
Sp. gr.	0.933 at 15°
Reichert-Meisel value ...	2.11
Solidified at	-5°

These values are closely similar to those given by Schuette and Enz [1931] and Schuette *et al.* [1930] for the expressed and residual oils from the endosperm of the Brazil-nut, except that the shell-oil has a somewhat higher Reichert-Meisel value suggesting that it had become slightly rancid during storage of the shell before use.

The crude extract was saponified and the fatty acids separated by the lead salt process. The unsaponifiable residue was negligible in quantity. After repeated crystallisation the "solid" lead salts retained an appreciable quantity of the "liquid" fraction.

The "solid" acids had equiv. by titration 236 and I.V. 12 and the "liquid" acids equiv. by titration 187 and I.V. 131.5, figures which again indicate the essential identity of the shell-oil with the endosperm oil examined by Schuette *et al.*

It is probable therefore that the oil in the shell is a casual intrusion by diffusion from the endosperm and has no part in the structure of the lignified tissue which forms the shell.

Alcohol-soluble fraction.

The dark brown amorphous mass was readily soluble in pyridine and partially soluble in water leaving a brown, gummy residue. The water-soluble part gave a green colour with methylene blue, indicating the presence of tannins. Further,

the green colour with ferric chloride and the yellowish brown precipitate with bromine confirmed the first test and indicated that the tannins were of the catechol type. Further investigation of this fraction yielded no other conclusive results.

Water-soluble fraction.

Part was dissolved to a strong solution, but fractional precipitation was of no value in the isolation of any of the constituents. An aqueous solution of the fraction, decolorised with charcoal, was acid in reaction and failed to reduce Fehling's solution. The solution was reduced to a small volume *in vacuo* and on standing deposited crystals which, on dissolving in water, gave a precipitate with lead acetate solution, and on testing with resorcinol and sulphuric acid gave a reddish tint and on heating a deep violet colour. They were therefore considered to be tartaric acid.

5% NaOH-soluble fraction.

The three fractions hemicelluloses A, A 1, B, were brown or fawn-coloured amorphous bodies. A and A 1 made up the major part of the alkali-soluble fraction, B being present only to the extent of 1%. A was partially soluble in warm water and was divided into water-soluble and insoluble fractions, the former being separated by acidification; the acid-insoluble precipitate was collected and dried by a sequence of alcohols of increasing strengths.

The quantitative determinations were as follows.

(1) *Lignin content.* The hemicellulose was treated with fuming HCl for 18 hours, the acid diluted to 5% and boiled for 6 hours. The residue washed free from acid was regarded as lignin.

(2) *Furfuraldehyde yield.* The phloroglucide precipitation method was used. The precipitate was washed with hot absolute alcohol to remove methylfurfuraldehyde and ω -hydroxyfurfuraldehyde. In view of the confused state of the literature as to the method to be used to obtain accurate results, this procedure is followed without any claim being made concerning the accuracy of the results obtained, but at least minimum furfuraldehyde yields can be calculated.

(3) *Uronic anhydride content* by the established carbon dioxide yield method.

The identification of the acids and sugars contained in these fractions was based on their acid hydrolysis products. The hemicelluloses were hydrolysed with 3% H_2SO_4 for 6-8 hours. The residue was filtered off and the filtrate half-neutralised with barium hydroxide and completely with barium carbonate. The precipitate which came down was filtered off and will be referred to as precipitate 1. The filtrate was reduced *in vacuo* to a small bulk and treated with three volumes of alcohol. Any precipitate which came down was filtered off and will be referred to as precipitate 2. The filtrate was evaporated to dryness *in vacuo* and extracted twice with hot absolute alcohol, then with 80% alcohol and finally with water. These solutions were evaporated to dryness, taken up in water and decolorised with charcoal.

In no case was the attempt to isolate the uronic acid successful; the small quantity present was probably decomposed by the hot acid during the hydrolysis. In two cases a trace of pentose was found among the sugars and it is possible that this represents the remains of a decarboxylated uronic acid.

	Hemicelluloses			
	A		A 1	B
	Water-soluble %	Water-insoluble %	%	%
Lignin content	70.8	41.0	67.5	34.5
Uronic anhydride	8.16	9.2	11.24	18.4
Furfuraldehyde	1.8	3.1	4.6	6.2

Hemicellulose A.

Water-soluble. Precipitate 1 on decomposition with dilute H_2SO_4 yielded no identifiable products. Precipitate 2 was very small in quantity. In aqueous solution it gave barium reactions with H_2SO_4 and with an aqueous solution of rhodizonic acid. (The latter is a micro-test using a freshly-prepared 1% aqueous solution of the acid which gives a brown stain when in contact with barium salts on filter-paper.) The Ba content of this salt, after being twice precipitated with alcohol from solution in water, was 39.6%. The amount of the salt was too small to permit of further examination.

The absolute alcohol extract gave a faint pentose reaction with orcinol and HCl but the quantity was too small to permit of identification of the sugar. The aqueous extract, treated in the usual way, yielded mannosephenylhydrazone, m.p. 190° .

Water-insoluble. Precipitate 1 contained no organic matter. Precipitate 2 was a barium salt (identified as before) having Ba 30.7%, but obtained in too small quantity to permit of any examination of the acid radical.

The absolute alcohol extract gave a strong pentose reaction with the reagents previously used and on treatment with bromine and cadmium carbonate the characteristic crystals of cadmium xylobromide were obtained. The water-soluble fraction reduced Fehling's solution and yielded glucosazone, m.p. 203° .

Hemicellulose A 1.

Precipitate 1 contained no organic matter. Precipitate 2 was decomposed with dilute H_2SO_4 in the calculated quantity. The precipitate was removed and the filtrate concentrated *in vacuo*. Part was neutralised with barium hydroxide, yielding a crystalline barium salt with Ba 25.5%. Another part was warmed and neutralised with cinchonine. The resulting cinchonine salt crystallised from 90% alcohol melted at 186° . A third portion was evaporated to a gummy mass on a boiling water-bath; when this syrup was kept in a vacuum desiccator over H_2SO_4 for a long time, crystals separated which, after freeing from the residual gum, melted at 130° . Gluconic acid forms a crystalline salt containing 26.0% Ba, forms a cinchonine salt, m.p. 187° , and on keeping in a desiccator for a long time forms a lactone, m.p. $134-136^\circ$.

The absolute alcohol extract gave a pentose reaction and on treatment with the usual reagents formed crystals of cadmium xylobromide. The 80% alcohol extract gave a pentose reaction and on treatment with *p*-bromophenylhydrazine hydrochloride and sodium acetate in the cold gave a crystalline mass which when recrystallised from 60% alcohol melted at 160° . The corresponding derivative of arabinose melts at 162° .

Hemicellulose B.

This was a dark brown amorphous solid with a lignin content of 34.5%, a uronic anhydride content of 18.4% and a furfuraldehyde yield of 4.2%. The amount available was too small to permit of further analysis.

Cellulose fraction.

This was prepared from the lignocellulose residue of the 5% NaOH extraction. This residue was washed free from alkali, dried and treated for periods of 5 min. with gaseous chlorine. After each chlorination the excess chlorine was removed by washing with brine and the chloro-derivative removed by boiling either with sodium sulphite solution or 95% alcohol. The process was repeated until cellulose

was obtained free from lignin. The resulting cellulose was a hard, greyish mass with a uronic anhydride content of 4.68 % and a furfuraldehyde yield of 17.4 %. Allowing for the furfuraldehyde from the uronic acid, the pentosan content was high and, using Kröber's tables, was calculated as 29.3 %. The pentosan was extracted from the cellulose by boiling it with 12 % NaOH for 5 hours. The residue was filtered off, the filtrate cooled and treated with an equal bulk of 95 % alcohol at 35°. The precipitate accumulated overnight and was filtered off as a granular mass. The pentosan was hydrolysed by the method of Heuser and Jayme [1923] by boiling it with 3 % HNO₃ for 1 hour, the solution neutralised with barium hydroxide and evaporated to dryness *in vacuo*. The dry mass was extracted with boiling absolute alcohol three times. The alcohol was removed, the residue taken up in water, decolorised with charcoal, the colourless solution was evaporated to dryness *in vacuo* and the resulting mass crystallised from absolute alcohol. The crystals after twice recrystallising from absolute alcohol melted at 142°. With bromine and cadmium carbonate crystals of cadmium xylobromide were obtained. The cellulose fraction appears to be associated with a large amount of xylan.

Lignin fraction.

The lignocellulose residue from the 5 % NaOH extraction was used in this preparation. An attempt was made to isolate the lignin as a chloro-derivative by treatment with gaseous chlorine, removal of the excess by washing with brine and extraction of the derivative with 95 % alcohol. The chloro-derivative was obtained from the alcoholic solution by precipitation with water. This method was unsuccessful, the lignin having undergone oxidation and having a variable chlorine content. Acid hydrolysis however yielded a uniform product. The dried lignocellulose residue was treated with fuming HCl for 18 hours, the solution diluted to 5 % and boiled for 6 hours. The residue was washed free from acid and on combustion gave the following figures, C, 58.62; H, 6.63 %. C₉H₁₂O₄ requires C, 58.70; H, 6.63 %. Treatment with acetic acid and acetic anhydride containing a few drops of sulphuric acid for 1 hour in a boiling water-bath yielded no acetyl derivative, the lignin being recovered unchanged. Benzoylation either in alkali or pyridine resulted in a complex change which obscured any addition of benzoyl groups. This change has been observed in the attempted benzoylation of lignin from other sources. By the semi-micro method of Clarke [1932] the product gave 3.2 % methoxyl.

DISCUSSION.

As already suggested the fat content of the shell is probably a casual intrusion from the endosperm where it is the main reserve product of the seed. The hemicellulose fractions are similar in constitution to those found in other plant tissues, with the exception that acids of the gluconic type have not previously been isolated, uronic acids being the usual form. The furfuraldehyde-yielding bodies with the exception of uronic acids are present only in small quantities in this fraction and in no wise compare in quantity with the 5 % of pentosan not in pulp which Emley [1928] found in the peanut hull. The cellulose however with its large xylan content is similar to that from the peanut hull. The uronic acid content is much higher than that found in other lignified tissues. The greatest precautions were taken to avoid over-chlorination and consequent cellulose oxidation and exclude any possibility that the high uronic acid value was a result of the method of cellulose preparation. In view of the hardness of

the shell it is interesting to note that the cellulose was obtained as a hard mass whatever method of drying was used. This can be compared with the cellulose obtained from timber after the attack of the dry-rot fungus, *Merulius lacrymans*. In this case the cellulose was isolated as a hard grey mass with a high uronic acid content [Barton-Wright and Boswell, 1931]. There appears to be a close relationship between the uronic acid content and the texture of the cellulose obtained. The lignin basic formula of $C_9H_{12}O_4$ fits into the C_{36} formula which appears to be the basis of lignins isolated from many sources. Owing to the unreactive and insoluble nature of the substance it is extremely difficult to obtain even an approximate value for its mol. wt.

This investigation has shown that these two nutshells, peanut and Brazil, have certain characteristics in common (pentosan content) and other shells are being examined to determine to what extent the common ground can be extended.

SUMMARY.

Brazil-nutshells reduced to a fine powder were extracted with benzene, alcohol, water and 5 % NaOH successively, lignin and cellulose being prepared from the residue. These fractions were examined and their constituents identified as far as possible. The hemicellulose and lignin fractions were similar to those isolated from other lignified tissues. On the other hand the cellulose fraction with its high furfuraldehyde yield was similar to that of peanut hull. The fat isolated from the benzene extract was identical with that isolated by other workers from the endosperm.

My thanks are due to Messrs Rowntree of York for their kindness in supplying the nutshells, to the Trustees of the Dixon Fund of the University of London for the use of the micro-balance required for the combustions, to Prof. J. W. Edington for the use of the centrifuge which so greatly reduced the time and labour required for the preparation of the slimy hemicellulose fractions and finally to Prof. B. H. Bentley for his interest in this work and the many ways in which he has facilitated it.

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CXLI. THE CHEMICAL COMPOSITION OF TEETH.

III. THE VARIATIONS IN CHEMICAL COMPOSITION IN RELATION TO DENTAL STRUCTURE.

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DIFFERENT theories exist as to the causes, remote and immediate, of dental caries, but it is probable that all theories admit of the view that the more perfect the structure of a given tooth, other things being equal, the less likely is that tooth to become carious. The relation between surface and histological structure of the teeth and the incidence of caries was investigated in children by Mellanby [1923; 1927, 2; 1934]. It was shown by her that when teeth were graded either in the hand or in the mouth for both the degree of hypoplasia and of caries, among the teeth which showed the more severe hypoplasia there was a greater incidence of caries. Hypoplastic teeth [Mellanby, 1927, 1] are rough and have pits and fissures, as opposed to perfect teeth which have a hard, smooth surface. It is easy to understand that the former would more easily harbour and favour the growth of the micro-organisms of caries; but the relationship between hypoplasia and caries might go farther than this and be in some way related to chemical composition or structure.

Thewlis [1934], as a result of X-ray analysis of human teeth, concluded that there were, judged from this particular physical aspect, three kinds of enamel, exhibiting variations in the amount of preferentially orientated apatite and in the degree of preferential orientation. Whether these classes of enamel can be related to the degree of imperfection of the surface structure, further work must decide. The aim of the present investigation was to see whether chemical analysis could establish any differences between perfect and imperfect (hypoplastic) non-carious teeth. The analyses were carried out on material obtained from the L.C.C. school dental clinics. All the extractions from 12 clinics were collected over a period of nearly 2 years. The total number of teeth obtained was about 10,000. The sound premolars (numbering over 300) were selected and carefully classified by examination with a lens and probe. Five classes were formed. The figures for the percentage occurrence of the teeth of the different classes are given.

		Occurrence %
(1) Class N	No hypoplasia, or normal	4
(2) Class Hy	Slight restricted hypoplasia	26
(3) Class Hy +	More generalised hypoplasia	36
(4) Class Hy + +	More severe and more generalised	23
(5) Class Hy gross	Very severe, areas deficient of enamel	11

In classifying these teeth the method used was that of Mellanby [1927, 1]. She, however, graded into four classes and included a class Hy— which would correspond to Hy or come between N and Hy in the above classification.

It must be remembered that these figures do not apply to all teeth but to teeth of this one group, sound premolars, removed from children under 14 years of age for regulation purposes. The reason for the small percentage of non-hypoplastic (class N) teeth is probably that perfect teeth do not often occur in

an overcrowded mouth. Teeth not fitting well into this classification, for example a few showing severe but very restricted hypoplasia, were discarded. Analyses of the separate enamel and dentine obtained as previously described [Bowes and Murray, 1935, 2] were made. Classes 2-4 inclusive were analysed quantitatively for ash, N, Ca, Mg, P, CO₂ and Cl by methods described in a previous communication [Bowes and Murray, 1935, 2], in which was set forth the chemical composition of the class Hy. In that communication references to methods and to previous investigations were given. So few perfect teeth were obtained that the separate analysis of class N had to be abandoned—(a few N teeth were included in the analyses of class Hy). Sodium and fluorine were not determined in all classes because of the relatively large amount of material required. Small variations in the amounts of these two elements are probably not significant in this particular group of analyses. Fluorine has not been included in the calculations because it is possibly not present in all teeth.

Mellanby [1929] noticed that on ashing in a crucible the teeth of dogs fed on different rachitogenic diets, the less well calcified teeth gave a black or grey ash, whereas the teeth of good structure gave a whiter ash. The weight of ash and the Ca content calculated as percentage of "wet" weight were less in the hypoplastic teeth of rachitic animals. The children from whom the teeth used for the analyses in the present investigations were obtained were not examined for evidence of past rickets, but since hypoplasia (gross) of the teeth is associated therewith [Wilson and Suric, 1930; Eliot *et al.*, 1933; Mackay and Rose, 1931], it might be presumed that the more severely hypoplastic teeth came from children who had had rickets. These human teeth, then, formed to some extent a series comparable with those of Mellanby's dogs. It was therefore of interest to repeat on these classified permanent teeth what might be called a rough test of hypoplasia. The teeth used for this purpose were all second maxillary premolars and of as nearly the same weight as possible. This was considered important because it was hoped in this way to minimise differences in modelling of the teeth and in the relative amount of enamel and dentine. The teeth were taken as soon after extraction as possible, having been kept moist, and were placed in saline in a refrigerator, if not used at once. They were brushed vigorously, the crowns cut off at the gingival line and the pulp cavities cleaned out. The crowns were dipped in alcohol and dried between filter-paper and put in an oven at 75° for a few minutes to remove the alcohol. They were weighed in a crucible, heated at first over a free flame and then with a blow-pipe till all smell of burning had gone; at this stage differences in the colour of the ash were noticed similar to those observed by Mellanby. Longer heating in a furnace, however, converted all samples into a white ash. Frequently this ash had a pinkish tinge. The final weights of the ashes were then taken and determinations of Ca made, the following results being obtained.

Class	Initial "wet" weight (g.)	Weight of ash (g.)	% Ash	Ca % in "wet" material
Hy	0.6698	0.5691	84.98	31.62
"	0.6188	0.5229	84.50	32.64
"	0.5628	0.4780	84.92	33.00
Hy +	0.6068	0.5084	83.79	30.42
"	0.5801	0.4791	82.57	31.36
"	0.6809	0.5590	82.10	30.96
Hy + +	0.6084	0.4942	81.23	29.73
"	0.6612	0.5402	81.7	29.65
Hy gross	0.4700	0.3665	77.98	29.07
"	0.5186	0.4127	79.58	29.71

The small but progressive decrease in the ash and Ca content from class to class represents a decrease in calcified material and indicates, either a relative decrease in the depth of the enamel, or a relatively greater organic content of the dentine, or a greater total area of poorly calcified areas and interglobular spaces in the dentine. It was found impossible to select teeth of the gross class of a weight equal to that of the others, no doubt because of the areas deficient in enamel; the enamel has a greater specific gravity than dentine. It would probably have been more satisfactory if slices of fresh dentine had been used in this test.

The composition of different enamels. The results of the detailed analyses of the enamels of the different classes are given in Table I.

Table I. *Analysis of enamels.*

% in dry enamel	Hy	Hy +	Hy + +	Hy gross	Apatite (carbonate)
Ash	95.38	95.20	94.76	94.67	---
N	0.156	0.161	0.153	0.208	---
Ca	37.07	36.85	36.29	35.81	---
P	17.22	18.01	18.04	17.72	---
CO ₂	1.952	2.312	2.424	2.434	---
Mg	0.464	0.564	0.404	0.477	---
Cl	0.3	0.334	0.29	0.19	---
CO ₂ % in ash	0.54	0.638	0.584	0.665	---
Ca : P : CO ₂ (molar)	10 : 5.99 : 0.48	10 : 6.31 : 0.57	10 : 6.41 : 0.61	10 : 6.39 : 0.62	10 : 6 : 1
Ca/P	2.153	2.046	2.012	2.020	2.151
Ca/CO ₂	18.99	15.94	14.97	14.71	9.10
P/CO ₂	8.8	7.8	7.6	7.6	4.23

Determinations of Na and K were made in one class only. Hy had 0.25 % Na and a trace of K; the latter was not found spectroscopically.

Consideration of the figures makes it possible to draw several conclusions. Firstly the composition of the different enamels is very similar, which fact has in the past made many workers say that it is constant. But close consideration reveals differences. The loss in weight on ashing increases with increasing hypoplasia, giving a decreasing ash content. This could result from an increased content of combined CO₂ or H₂O, or organic matter. There is definitely an increased CO₂ content. The small decrease in Ca content with increase of hypoplasia is in keeping with the decrease in ash content. The phosphorus value does not vary in the same way but shows both a relative and an absolute increase with a consequent effect on the Ca/P ratio. For this reason it is to be emphasised that no analyses of the Ca content of tooth substance should be considered apart from the phosphorus content. The progressive decrease in Ca/P ratio and the consequent departure from the apatite value is considered by us to be one of the most significant points as regards the enamel constitution. The Mg values do not suggest that where the ratio is lower Mg has replaced Ca, for with the exception of the Hy+ class the Mg values show little variation. This would indicate that the increase in Mg content found in caries and pyorrhoecia by various workers [Howe, 1926; Ulrich, 1925; Kaushansky, 1932] is a result and not a predisposing cause of the condition.

The amount of CO₂ in enamel does not permit the assumption that carbonato-apatite is the only compound present. The chlorine value decreases progressively from the sound to gross hypoplasia. The significance of this is hard to determine. Fränkel [1907] stated that with increasing age there was a decrease in halogen content but we are not dealing here with age differences. The highest chlorine

value we have obtained was found in the enamel of sound molar teeth of old people, natives of India. All ashed samples of enamel have a small CO_2 content, the amount does not seem to vary much. The variations in the molar proportions are significant and show a progressive increase in phosphorus and CO_2 .

In order to bring out points of difference between the different classes of teeth, calculations have been made to arrive at some idea of composition of these various enamels. Any calculations of composition are beset with difficulties because of lack of precise knowledge as to the manner in which the inorganic substance is laid down. It is generally accepted that various X-ray investigations have settled the fact that the chief constituent of bones and teeth is a form of apatite. But the existence in teeth of various cations and anions not present in calcium carbonate or hydroxy-apatite necessitates the consideration of the important point discussed fully by Robison [1932]. Robison's view is that, in the absence of more knowledge of the physicochemical system of calcifying tissues, it is dangerous to postulate the composition of the calcified substance. He prefers the view based on much experience that there is one bone and tooth compound, fundamentally a complex calcium carbonato-phosphate in which the Ca can be replaced by equivalent amounts of Mg, Na or K and in which the carbonate moiety can be replaced by $(\text{OH})_2$ or Cl_2 and occasionally by minute traces of F_2 . Such a replacement theory might explain the alterations in composition of bones with age and in fossil bone formation. In any case it must be admitted that from such a complex selection of ionic substances as exist in blood plasma, the possibility that the calcified substance will contain some of all the ions present is very great. Whether these other ions like Mg^{++} and Cl^- etc. are actually present combined in the bone compound or whether adsorbed or separately deposited has not been determined.

A detailed consideration of the results obtained on the enamel and dentine from the different grades of teeth has been made. One of the points considered was the calculation of the total cation (Ca^{++} , Mg^{++} , Na^+ and K^+) and anion (PO_4^- , CO_3^- , Cl^-) concentrations. The following values were obtained (Table II).

Table II. *No. of cation and anion gram equivalents per 100 g. enamel.*

	Hy	Hy +	Hy + +	Hy gross
Cation	1.9032	1.901	1.8592	1.8413
Anion	1.7694	1.8564	1.8702	1.8374
Excess cation	0.1338	0.0446	- 0.0110	0.0039

There is then a progressive decrease of total cation and a less regular diminution of anion concentration. The better formed enamel is more basic.

If all the cations are calculated as equivalent Ca and compared with the P and CO_2 values, then only in the Hy grade can all the P be present as apatite. In no grade of enamel is all the apatite in the carbonato-form; some hydroxy-apatite must be present. We have calculated the possible composition of the different enamels, not so much to postulate the composition as to make comparisons between the different classes.

The Mg has been included in the apatite and where possible all the P has been placed as apatite. When an excess of Ca occurred it has been included as CaCO_3 and where an excess of P as CaHPO_4 . The inclusion of the latter compound must be taken as provisional until a more satisfactory means of dealing with the excess P has been found.

This method of calculation gave the figures set out in Table III.

Table III.

	Hy	Hy +	Hy + +	Hy gross
Chloro-apatite $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaCl}_2$	4.397	4.91	4.251	2.768
Carbonato-apatites $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaCO}_3$ $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{MgCO}_3$	24.498	53.73	56.47	56.64
Hydroxy-apatite $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$	64.51	33.81	26.47	27.99
Excess Ca as CaCO_3	2.028	Nil	Nil	Nil
Excess P as CaHPO_4	Nil	4.803	9.342	7.675

Such a table emphasises the effect which small differences in Ca and P have on the calculated composition. Facts which could be deduced from the anion and cation values are shown in this table. The diminishing total apatite, the increase of carbonato-apatite and the more acidic phosphorus compound as the hypoplasia increases are the notable points. Because of the different molecular weights of the different apatites it is better to compare the apatite calcium. If the value for the apatite P of Hy is put as 100, then the corresponding values for the other classes are: Hy + 98, Hy + + 92.5, Hy gross 92.5. The calculations then seem to bear out the deduction made earlier that enamel of the hypoplastic teeth contains less apatite.

Dentine. The analyses of the dentines never gave such consistent results as did those of the enamels. The inconsistencies encountered in the dentine are probably mainly due to the fact that even after eruption, and particularly soon after, new dentine is being laid down. This later and post-eruptive dentine deposited near the pulp, possibly because it is more slowly deposited, is often of better structure than the rest in a hypoplastic permanent tooth [Mellanby, 1927, 1]. In the collection of the dentine for analysis, some of this more highly calcified, as well as some of that near the enamel, which is often defective, was not included, so that though the dentine of the teeth of each class would be largely of good structure, nevertheless it can be seen that dentine can never be uniform throughout except in perfect teeth. The most characteristic differences between the dentine of teeth of good and bad structure is to be seen by histological investigation, showing the occurrence of interglobular spaces. But if there are differences in the composition of the calcified material, then our method should detect them. Determinations were carried out on dry and ashed dentine and the results given in Table IV were obtained. Sodium and potassium were not estimated in the separate classes. Class Hy + dentine contained 0.19% Na and 0.07% K. These values were used in calculations.

Table IV. *Analysis of dentines (% in dry dentine).*

	Hy	Hy +	Hy + +	Hy gross	Apatite (carbonate)
Ash	71.09	70.64	70.17	70.28	—
N	3.43	3.247	3.371	3.449	—
Ca	27.79	27.54	27.27	26.96	—
P	13.81	14.35	13.61	13.5	—
CO_2	3.176	3.383	3.046	3.102	—
Mg	0.835	0.882	0.797	0.728	—
Cl	Nil	Nil	Nil	0.023	—
Ca : P : CO_2 (molar)	10 : 6.12 : 1.04	10 : 6.72 : 1.12	10 : 6.44 : 1.02	10 : 6.46 : 1.03	10 : 6 : 1
Ca/P dry	2.012	1.919	2.004	1.995	2.151
Ca/P ash	2.091	2.044	2.059	2.043	—
Ca/ CO_2 dry	8.75	8.13	8.95	8.89	9.10
P/ CO_2 dry	4.15	4.24	4.46	4.35	4.23

The results show less than the enamels in the way of progressive alteration of any one constituent. Slight decreases in ash, Ca, P and in the Ca/P ratio occur with increase of grade of hypoplasia. There was no increase in the Mg as the hypoplasia increased. This is worthy of note since an increase in Mg in whole crowns occurs in both caries and pyorrhoecia [Ulrich, 1925; Kaushansky, 1932]. It is probable that the low Mg in gross hypoplasia is a general occurrence, for we have obtained several low figures for this value. The Ca/P ratios for all dentines, even after ashing, are less than that required by apatite. A comparison of the enamel and dentine values should be made, since many people consider them to be identical in all respects but organic content. The differences found were: (1) The Ca/P of ashed dentine (average) is 2.057, which is lower than the average enamel value of 2.140. (2) Dentine contains twice as much Mg as enamel. (3) Dentine has a higher CO_2 content, all of which is driven off by ashing. (4) The dentine substance, with one exception, does not contain combined chlorine. Dentine probably contains NaCl in interglobular spaces and lymph channels in its nourishing liquid, but this would have been washed out with water and all these Cl determinations, like those of sodium, were made after water extraction, in a dilute HNO_3 solution. The sodium content of dentine (not listed) was only 0.19%.

The close correspondence of the dentine with carbonato-apatite seen in the molar proportions in Table III, is only apparent because no correction has been made for the Mg, which must make a considerable difference. Calculations of the probable composition of the dentines presented great difficulties. Having tried many methods we came to the conclusion that in dentine also apatite is the chief constituent and that more of this is in the carbonato-form. Other substances appear to be present, for instance CaCO_3 or MgCO_3 and an acidic phosphate compound. It was considered best to deal with anion and cation concentrations as a basis for comparison. These are set forth in Table V.

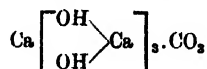
Table V. *No. of cation and anion gram equivalents per 100 g. of dentine.*

	Hy	Hy +	Hy + +	Hy gross
Cation	1.4692	1.4606	1.4400	1.4188
Anion	1.4854	1.5468	1.4594	1.4521
Excess anion	0.0162	0.0862	0.0194	0.0333

Hence it is seen that whereas in enamel there is in most cases excess of positive equivalents, in dentine there is always an excess of negative equivalents. This fact and the Ca/P ratios point to the existence of some form of calcium phosphate other than tricalcium phosphate, a more acidic phosphate. Burns and Henderson [1935] came to a similar conclusion working with bone. Even if all the metals are expressed as the equivalent amount of Ca and the "corrected" Ca/P obtained, these ratios do not reach the apatite value. The corrected Ca/P ratios are: Hy 2.126, Hy+ 2.034, Hy++ 2.114, Hy gross 2.101. As to the comparison between the classes, the best class is the most basic and gives the highest Ca/P both actual and corrected. That is, the best dentine is a more basic dentine as the best enamel is also more basic.

A chemical theory of the causation of caries was put forward by Gassmann [1921]. He studied wisdom teeth and compared them with other teeth; his conclusion was that whereas in bones and teeth generally, the proportions of Ca : P : CO_2 were always 10 : 6 : 1, in wisdom teeth there was excess Ca and CO_2 over and above proportions. This excess Ca and CO_2 was in the proportion 4 : 1, which he took to mean that the formation of apatite was incomplete.

His theory was that the first formed compound in calcification was the co-ordination compound



called the "hexol salt", this by replacement of the hydrogen of the hydroxy-groups by CaPO_3^- was converted into carbonato-apatite. The hexol salt was less stable, *i.e.* less resistant than apatite, so that when incomplete conversion took place, susceptibility to caries resulted. His argument was indirect, based on the known susceptibility of wisdom teeth to caries. The point of interest here is that the results put forward by the present authors indicate that hydroxy-apatite (as well as the carbonato-apatite) represents an abundant compound in the enamel of teeth. There is lack of agreement between us and Gassmann, in that his theory would correlate susceptibility to caries with a relative diminution of phosphorus, whereas we find invariably a relative increase of phosphorus with increase of hypoplasia.

In conclusion it would be well to emphasise that the results have shown that teeth do vary in their chemical composition but that these variations can only be seen in rather elaborate analyses of separated enamel and dentine. This investigation confirms by chemical evidence the X-ray findings that different kinds of enamel exist [Thewlis, 1934] and indicates that X-ray analysis of different classes of enamel should give significant results. The analyses are being extended to carious and pyorrhetic teeth.

SUMMARY.

1. Analyses of separated enamel and dentine of teeth showing the different grades of hypoplasia defined in this paper were made. The determinations included ash, N, Ca, Mg, Na, K, P, CO_2 and F.

2. Small progressive variations in P content, Ca/P ratio and CO_2 content occurred.

3. With increase of hypoplasia the enamel showed definite alterations of composition, the most outstanding of which was the decrease of apatite content from 92 to 86 % and a corresponding increase of some other phosphorus compound.

4. The different dentines showed less regular variations in composition. Alterations in composition of the inorganic part, similar to those shown by the enamels, were found. The apatite decreased from 53 to 49 %.

5. Enamel and dentine differ in other respects than in the amount of organic substance, hence they should be analysed separately.

6. Calcium determinations alone are not sufficient to show up alterations of composition; they must be taken in conjunction with phosphorus and magnesium values.

We wish to record our thanks to the Medical Research Council for an expenses grant and for a personal grant to J. H. B. during the progress of this work.

We would also like to express our thanks and appreciation to the Sisters in charge of the L.C.C. dental clinics, for without their co-operation this work would have been impossible.

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CXLII. CRYSTALLINE TORULIN (AS VITAMIN B₁) AND THE INTERNATIONAL VITAMIN B₁ STANDARD.

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THE work described in this paper originated in 1931 with tests upon an acid clay supplied by Prof. Jansen as a preliminary trial for the international standard. It was continued with the actual international standard, and extended to include trials of extracts made from the international standard and of crystalline vitamin B₁. Both of the latter can be compared by injection and by the mouth. Some rather remarkable discrepancies have appeared between tests with our crystalline vitamin [Kinnersley *et al.*, 1935] and a vitamin concentrate.

EXPERIMENTAL.

Tests have been made by the technique previously described with little change. Birds have been left for at least 4 hours after dosing with sugar solution to minimise the number of pseudo-cures. Samples of clay or extracts have been given in approximately 5 ml. water, the latter being neutralised where necessary immediately before use.

We have made 3 types of comparison, (1) tests carried out over different parts of the year, (2) serial tests, in which a group of standard or other tests is sandwiched in between groups of other tests, and (3) some simultaneous tests, in which alternate birds are dosed with the preparations to be compared. Though the latter may give more confidence as a control, we are inclined to think that, owing to the particular way in which birds have to be prepared for these tests, the extra gain in control over the serial method is illusory [*v. Kinnersley et al.*, 1935, p. 707]. So far as we can tell, the variations as between one time of the year and the other lie within the error of the method. Following our usual convention we have not included in our reckoning those birds which were not cured for 1 day. Contrary to previous practice we have included in our average even very high tests, because it is difficult to exclude these: owing to the large error of the method big groups have been used.

Table 1 gives results for the solids, preparation 2 being the international standard. It is remarkable to note how consistent the values were for the groups of tests in 1934. The average value for the whole series of 50 mg. tests gives "10 mg. equal to 1.12 pigeon day doses in our terms".

Comparing Exps. 1-4 with Exps. 5 and 6, we see that high doses of the clay tend to give erroneous results. This is probably due to failure upon the part of the gut to elute the vitamin from the clay in the course of passage through the animal. R. B. Fisher (private communication) has found that food passes through the gut of pigeons in a few hours. Indirect evidence was obtained in some other experiments that this might happen.

There arises at once the question whether some of the variations in the figures of Table I arise from the uneven liberation of vitamin from the clay by the

Table I. *Jansen's activated clay.*

Exp.	Prep.	Year	Month	Dose mg.	No. birds	No days cured* av.	Not cured† %	Day dose mg.	Individual tests days
1	1	1931	Jan.-March	250	2	11.5	0	22.0	—
2		1931	Jan.-March	200	4	9.0	0		
3		1931	Jan.-Feb.	100	9	7.4	7.8	30	10, 4, 7, 6, 6, 13, 9, 7, 5 4, 5, 6, 7, 8, 15, 13
4		1931	March	100	7	8.3			
5		1931	May-June	50	5	5.6	5.1	20	6, 6, 6, 8, 2 5, 4, 5, 3, 2, 3, 5, 11, 9, 2, 4
6		1932	Oct.-Nov.	50	11	4.8			
7	2 (U.S.)	1932	Oct.-Nov.	50	8	6.5	—	7.7	4, 6, 6, 5, 7, 10, 10, 4
8		1934	28/29 May	50	10	5.5	9	9.1	10, 6, 2, 2, 3.5, 2, 15, 5, 5, 5
9		1934	Sept.	50	14	5.6	7	8.9	5, 5, 5, 3, 12, 2, 6, 5, 4, 8, 5, 9, 5, 4
10		1934	28 Oct. Nov.	50	18	5.3	—	9.4	5, 9, 10, 2, 5, 7, 4, 3, 5, 3, 6, 11, 2, 6, 9, 2, 3, 3
Average					50	5.6	—	8.9	1.12 average day doses 1.09 if 9.2 taken for 1934 figures

* Cure and protection.

† Not cured for 1 day; not included in results.

Preparation 2 is the International Standard (U.S.).

Serial tests. Exps. 6 and 7. Exps. 8 and 30-10 [c. Kinnersley *et al.*, 1935]: 34.3 immediately succeeded Exp. 10.

digestive juices. The matter has been tested by obtaining data with an eluate from the clay made with baryta. If the clay is merely shaken with the baryta as in the Jansen and Donath method, quantitative extraction from the clay does not take place. If, however, it is ground with baryta as is usual at the phosphotungstate stage in our preparation of vitamin B₁, results indicate that quantitative elution takes place.¹

Standard baryta extracts of the acid clay.

5.0 g. of the acid clay are ground in a 100 ml. centrifuge-tube with an equal volume of solid powdered baryta and 25 ml. distilled water for 3 min. at room temperature, 15 ± 3.0°. The whole is centrifuged and the extract filtered through a small filter-paper, the filtrate being made acid immediately with 20% H₂SO₄ avoiding excess. The residue is ground twice more with 25 ml. water, and the process repeated, in all 3 extracts being made. The combined filtrates are adjusted to pH 3.0, warmed, centrifuged and made up to a convenient volume containing 20% ethyl alcohol; it is suitable if 1.0 ml. is equal to 50 mg. of original clay.

Table II. *Extracts of activated clay.*

Exp.	Prep.	Year	Month	Dose mg.	No. birds	No. days cured Av.	Not cured %	Day dose mg.	Individual tests
11	1	1933	Jan.-Feb.	67	10	6.1	17	11.0	8, 14, 7, 7, 3, 2, 7, 4, 4, 5
12		1933	March-April	67	10	5.45	6.2	4	4, 4.5, 7.5, 5, 2.5, 6, 4, 9, 5, 7 6, 3, 2, 7, 10, 6, 7, 9, 9, 3, 6, 2, 9, 5, 13, 10
13		1933	18 April May	67	16	6.7			
14		1934	July 14-17	50	6	4.8	14	10.6	9, 4, 8, 2, 3, 3, .
15		1932/33	Nov.-Feb.	45	3	4.3	—	10.5	4, 4, 5
16		1932/33	Nov. Feb.	22.5	5	3.8	—	5.9	2, 2, 5, 2, 8
15	2 (U.S.)	1932/33	Dec.-Jan.	72.6	7	7.3	—	9.9	6, 5, 10, 9, 2, 10, 9
16		1932/33	Dec.-Jan.	54.5	7	7.0	12	7.8	4, 5, 9, 11, 9, 5, 6
17		1932/33	Dec.-Jan.	36.3	4	7.2	20	5.0	4, 4, 5, 16
18		1932/33	Dec.-Jan.	18.2	6	2.8	—	6.5	4, 2, 2, 5, 1, 3
19		1934	June	50	15	6.0	18	8.3	3, 10, 5, 2, 5, 11, 1, 10, 5, 11, 6, 7, 4, 5, 5

¹ We do not know whether this has advantage over the quinine method of Williams *et al.* [1934].

In Table II are given all the results obtained with the extracts; in Exps. 15, 16, 17, 18, there are not enough birds for the results to be more than suggestive, but there is a tendency for high results to be obtained with amounts below the equivalent of 50 mg. In our hands, less than 50 mg. did not cure many birds when it was given as the solid. There is no more than this in favour of the improved rate of absorption of the extract; for in Table III it will be seen that the results obtained with the solid and with the extract are remarkably alike and well within the limits of error.

Table III. *Comparison of tests with solid and extracts. (Activated clay.)*

Prep.		Amount mg.	No tests	Day dose mg.
1	Solid	50	16	9.8
	Extract	50-67	22	10.7
2, i.s.	Solid	50	50	8.9
	Extract	50-73	29	8.5

It may be enquired further whether the injected extract of the clay compares favourably with the extract given by the mouth.¹ The experiments are shown in Table IV. Experiments with the i.s. were sufficient to show the correspondence.

Table IV. *Comparison of injection with oral administration (50 mg. dose).*

Exp.	Prep.		Year	Month	No birds	No. days cured, av.	Not cured %	Day dose mg.	Individual tests
14	1	Mouth	1934	July	6	4.7	14	10.6	See Table II
20		Injection	1934	July	6	3.5	Nil	14.0	4, 4, 3, 4, 3, 3
19	2	Mouth	1934	June	15	6.0	18	8.3	See Table II
21		Injection	1934	June	22	6.05	—	8.3	6, 4, 3, 8, 10, 6, 4, 3, 4, 5, 4, 1, 11, 4, 5, 6, 7, 6, 4, 13, 13, 6

In each case tests were simultaneous.

This is important because it gives confidence in the oral route for administration: but further it makes it possible with the baryta extracts to have a soluble standard for injection, made quantitatively from the i.s. So far the impossibility of injecting the i.s. of vitamin B₁ has been an objection. It may be significant that all birds were cured with injected vitamin, as compared with 84 and 88% by mouth.

The day dose method and crystalline vitamin B₁.

The international Committee has asked us (and others) to determine the relation between the activity of crystalline vitamin B₁ and the i.s., for which 1 unit is 10 mg. We did not anticipate that this would prove an awkward task, but actually it is beset by considerable difficulty with the pigeon, to which some brief allusion has already been made [Kinnersley *et al.*, 1935]. The experiments in this laboratory have been based upon the assumption that there is a satisfactory relation between the time of cure and the dose given. Others are in agreement with this view among whom we may mention Coward *et al.* [1933], Birch and Harris [1934] and Ammerman and Waterman [1935]. The tests in Tables I and II do not bring this out very clearly though the rule seems to be

¹ Our previous view that injected vitamin is more potent than vitamin by mouth is not supported in this paper for these extracts; but for crystals there was slight evidence in its favour [Kinnersley *et al.*, 1935].

followed roughly if we exclude the very high and very low doses. Two further series of tests (see Table V) have been made with one of the 50% alcoholic extracts of charcoal prepared by us. The experiments in the first series tested were not simultaneous and not numerous, but the second series was done under

Table V.

Exp.	Year	Month	Dose ml.	No. of birds cured	No. days cured av.	Not cured	Day dose per 101 ml.	Individual tests	
A. Vitamin B ₁ concentrate 71 A.									
22	1932	April-May	0.05	8	4	1/9	8	2, 3, 1, 5, 6, 5, 6, 4	
			0.075	7	6		8	8, 13, 6, 3, 2, 4, 6	
			0.1	6	6		6	5, 7, 12, 3, 4, 4	
	1932	Feb.	0.25	9	9.3		3.7	10, 8, 10, 12, 9, 6, 10, 10, 9	
23	1935*	Mar.-July	0.05	36	4.9	8/44	9.8	Mar. 5, 5, 6, 4, 3, 2, 5, 5, 2, 5, 3 May 7, 11, 11, 4, 8, 2, 3, 4, 6, 6, 5, 3, 1 June 5, 5, 3, 10, 7, 6, 3, 3, 7, 5, 2, 4	
			0.1	37	7.85	9/46(av.)	7.85	Mar. 2, 6, 1, 4, 4, 9, 8, 5, 9, 5, 11 May 7, 5, 6, 11, 10, 16, 7, 3, 10, 17, 13, 6, 10 June 6, 4, 4, 6, 3, 6, 16, 5, 7, 13, 6, 23, 6	
			B. Crystalline vitamin B ₁ injected, 30.10.						
24	1934*		γ				γ		
		5	27	5.2	—	0.96†	10, 4, 5, 13, 4, 9, 8, 9, 5, 7, 3, 3, 6, 10, 3, 2, 3, 6, 2, 3, 1, 3, 3, 7, 2, 2, 6		
		10	23	5.7	—	1.6	11, 14, 5, 10, 4, 3, 10, 7, 2, 3, 7, 3, 11, 3, 3, 4, 3, 3, 12, 2, 1, 3, 8		
25	1934*	Sept.-Nov.	10	37	4.54	—	2.2	4, 2, 2, 3, 5, 6, 5, 3, 3, 5, 2, 5, 2, 2, 4 10, 7, 3, 3, 2, 6, 4, 7, 7, 2, 6, 5, 4, 3 Nov. 2, 3, 12, 7, 10, 1, 9	
		1934	Sept.-Nov.	15	42	4.55	—	3.3	7, 7, 5, 4, 4, 3, 3, 2, 5, 5, 8, 3, 4, 3, 5, 4, 4, 8, 8, 4 3, 2, 3, 4, 4, 5, 6, 4, 4, 5, 5, 3, 5, 4, 4, 4, Nov. 5, 3, 7, 8, 3, 5
		C. Crystalline vitamin B ₁ by mouth.							
26	1936	Jan.-Mar.	7	30	5.0	10	—	Jan. 3, 3, 0, 2, 3, 0, 7, 2, 4, 3, 5, 0, 0, 0 Mar. 2, 2, 0, 4, 4, 2, 2, 2, 6, 2, 0, 3, 0, 0, 18, 0, 15, 6, 2, 15, 7, 1, 4, 6, 13	
			14	37	5.8	4	—	Jan. 0, 3, 2, 4, 6, 3, 2, 5, 3, 0, 6, 5, 0, 9, 0 Mar. 6, 16, 2, 5, 4, 5, 7, 3, 1, 17, 7, 2, 4, 6, 6, 5, 2, 15, 5, 9, 3, 8, 3, 13, 10, 2	

* Simultaneous dosing.

† [Kinnersley *et al.*, 1935].

the best conditions of modern control. Doses of 0.05 ml. and of 0.1 ml. of the concentrate were compared over a period from March to July with simultaneous dosing. Though the relation is not rigid, the figures support the assumption which we have always made that vitamin B₁ in these concentrates shows a rough relation between dose and activity. Therefore it is all the more surprising to find that this relation does not hold at all for crystalline vitamin B₁; Table VB shows simultaneous tests, with different doses of injected hydrochloride.

The fact is that upon the average injected crystalline vitamin B₁ in our hands does not give a longer period of cure with increasing dose. Supposing that we are correct in believing that such a relation is shown by the concentrates, there are two possible explanations: (1) it is due to the route of administration or (2) it is due to some chemical difference, the impurities are having some adjuvant

action. We have tried to obtain light upon (1) by carrying out a series of simultaneous tests by the mouth with 7 γ and 14 γ vitamin, Table V c. In these again there is no clear relation between time of cure and dose, though there is a distinct tendency for the low dose not to cure the animals (so that the oral route is better for test). It may be interpreted that the difference is due to (2) and that there is some factor in the crude preparations which alters the effect of the vitamin B₁. This would be consistent with the suggestion of vitamin B₅, which has previously been made [Carter *et al.*, 1930], but the data are not decisive.

Meanwhile, it seems certain that the pigeon day dose is no use for deciding differences in potency between injected specimens of vitamin B₁. Fortunately this does not now matter because there are available both the colour reaction, formaldehyde-azo-test [Kinnersley and Peters, 1934], and the catatorulin test, as well as the bradycardia test which is stated to give good results.

Catatorulin tests (Appendix I) were made upon the extract of the international standard clay, comparing this with crystalline vitamin B₁. They functioned efficiently, and showed definitely that an amount of the international standard extract equal to 10 mg. of the original clay (1 unit) was equivalent in effect to 1.99 γ vitamin HCl.

Statistical considerations

As previously stated, a rough plotting of our figures shows that they have a skew distribution. If the values are plotted logarithmically, *i.e.* dose against the logarithm of the number of days of cure, there is a nearer approximation to the normal distribution. When the logarithmic averages are calculated with the standard deviation for some of the principal groups in this paper, the values of Table VI are obtained. These averages make no substantial difference in the

Table VI. *Results for the most significant experiments calculated logarithmically.*

Exp.	Dose	No. of birds	Mean (logarithmic) as days	Mean times lying between 21-22 days
7, 8, 9, 10	Activated clay 50 mg.	50	4.91	4.10-5.87
19	Activated extract 50 mg.	15	5.07	4.50-5.71
23	Vit. B ₁ concentrate	0.05 ml. 36 0.1 ml. 37	4.31 6.67	3.60-5.19 5.43-8.18
24	Crystalline vit. B ₁ injected	5 γ 27 10 γ 23	4.28 4.13	— —
25	Crystalline vit. B ₁ injected	10 γ 37 15 γ 42	3.85 4.3	3.22-4.60 3.84-4.81
26	Crystalline vit. B ₁ by mouth	7 γ 30 14 γ 37	3.76 4.68	2.96-4.78 3.76-5.82

Note. Figures in last column obtained by finding standard deviation (σ) for the logarithmic mean $\sqrt{\frac{\sum d^2}{n-1}}$ and then $\epsilon = \frac{\sigma}{\sqrt{n}}$. Actual figures = antilog mean $\pm 2\epsilon$.

conclusions, but the main contention is brought out more sharply so far as the difference between the crystalline vitamin and the 50% alcohol concentrates is concerned. The differences in Exp. 23 are significant. An application of Fisher's [1929] *t* test¹ (difference of means) gave $t=3.1516$ from which the probability that random sampling could give the differences is considerably less than 1 in 100. Compared with this in Exp. 26 (crystalline vitamin) $t=1.285$ giving a probability of 1 in 5. Provided that the statistical formulae apply to the logarithms, this may be considered to prove the presence of a chemical difference in the impure concentrate.

¹ We are grateful to Dr R. B. Fisher for this calculation.

DISCUSSION.

It seems from the catatorulin test that one i.u. (10 mg.) $\equiv 2\gamma$ vitamin B₁ hydrochloride; a similar value is obtained with formaldehyde-azo-tests, using the extract of the i.s. This value can be used to interpret the pigeon data. From Table VI we have 5 i.u. (50 mg.) $\equiv 5.0$ pigeon day doses, $5-15\gamma$ B₁ by injection and $7-14\gamma$ B₁ by mouth $\equiv 4.13$ and 4.38 day doses respectively. An approximately correct value is obtained for the crystals, if we assume that 10γ vitamin B₁ give the correct value. In this case 2.5γ B₁ would be equivalent to 1 i.u. In the past we have used mainly 10γ , and we have always quoted 1 i.u. as approximately equivalent to 2.0γ vitamin B₁. Hence the present work entails no modification of previous data. But it is now clear that the values reached previously with injected crystalline material were actually empirical.

In a recent paper from Suzuki's laboratory, Ohdake and Yamagashi [1935] have found for their vitamin hydrochloride $1.6\gamma \equiv 1$ i.u. At first sight this indicates a greater potency than ours. Examination of their protocols shows that they had a day curative dose for the i.s. of 15 mg., *i.e.* 50% more than needed by our pigeons. Their figures were (Table 1, p. 111),

International Clay dose mg.	No. of birds	Average day dose mg.
150	7	18.6
50	4	11.2

They averaged these figures in the belief that this was permissible with the adsorption product. From our Table 1, the higher amount of 100 mg. gave a larger average dose. If their results for 50 mg. are taken, they are so close to our own that there is no reason to believe in a higher potency. $0.0023-0.0025$ mg. was their actual day dose. That the crystals from Prof. Suzuki's laboratory are not more potent is suggested by two further facts. Heyroth [1936] has concluded that our preparation 64.19 by a rat technique is slightly more potent than one sent by Ohdake. We have carried out one catatorulin test (see Appendix) and colour tests upon a specimen of oryzanin fortior (Suzuki): the potency was identical with the specimen of our crystals which was used.

The biological explanation. A curious dilemma is raised by the finding that doses of $5-15\gamma$ vitamin show equal biological effects. The fact is difficult to reconcile with the well-known belief that utilisation of vitamin is proportional to the metabolic rate; for instance Cowgill's [1934] calculations for vitamin B₁ requirement are based upon the formula $\frac{\text{vitamin}}{\text{calories}} = k \cdot \text{weight}$. The explanation is likely to prove interesting.

SUMMARY.

1. One international unit of vitamin B₁ (10 mg. Jansen Clay) is approximately equivalent to 2γ crystalline vitamin B₁ hydrochloride.
2. Extracts of the International Standard clay of full activity can be made by a standard method and injected. The same result is obtained as by mouth.
3. The day dose method for pigeons is not satisfactory for assay of injected crystalline vitamin B₁, though useful for impure vitamin.

We are grateful to Messrs Wakelin and Clark for help with the tests, and to the Medical Research Council for a personal grant (H. W. K.) and for expenses. We also thank Prof. R. A. Fisher, for statistical advice and Miss Chick for the specimen of oryzanin fortior from Prof. Suzuki.

APPENDIX I.

Catatorulin tests.

These were done as described by Kinnersley *et al.* [1935], with the following changes: the Ringer-phosphate, p_H 7·3, contained Na pyruvate approximately 0·034 *M*. This was added immediately before tissue division with the glass

Table VII. *Catatorulin tests.*

Exp.	989	μl./g./hr.	992	1057
Vitamin B ₁ *	0	658	886	1018
	0·5γ	1020	1270	1540†
	1γ	1220	1322	—
I.s. extract	≅0·167 unit	923	—	—
	≅0·25 unit	—	1243	—
	≅0·333 unit	1088	—	—
Vitamin B ₁ (Suzuki)	0·5γ	—	—	1533†

By inspection of curve 0·167 i.c. = 0·35γ or 2·1γ per unit

0·333 i.c. = 0·65γ or 1·95γ per unit

0·25 i.c. = 0·48γ or 1·92γ per unit

Average = 1·90γ

* Vitamin B₁ (Kinnersley, O'Brien and Peters).

† Average of triplicate, rest duplicate.

crusher. Na pyruvate was made freshly before use by cautiously neutralising a solution of pyruvic acid (approx. 5 *M*) obtained from crystalline acid and kept in cold store. The reason for using pyruvate alone will be given in a subsequent paper by R. A. P.

Four experiments were made of which the details of two are given. The two not quoted were less satisfactory but in substantial agreement. The average O₂ uptake for the period $\frac{1}{2}$ –2 hours is given in Table VII.

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CXLIII. INVESTIGATIONS INTO THE PRESENCE AND THE RÔLE OF BROMINE IN THE BODY.

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THE elements of the halogen group being generally associated in nature (as in minerals, sea-water, mineral springs) are absorbed together by plants and animals and should be present as normal constituents of the living tissue. The presence of chlorine, iodine and fluorine in the body is known while that of bromine, denied for a long time, is doubted even in the latest editions of some biochemical handbooks.

In the past, some authors claimed to have demonstrated occasionally the presence of bromine in urine and tissues, but only a few workers employed a suitable technique. Damicus [1922] found bromine in the organs of dogs, and Bernhardt and Ucko [1925; 1926] showed that it was constantly present in the blood and organs of man.

Zondek and Bier [1932; 1933] pointed out:

(1) That the normal bromine content of the blood and the cerebrospinal fluid was greatly diminished during the different stages of manic-depressive psychoses.

(2) That the hypophyseal bromine diminished or disappeared during artificial sleep whereas the bromine content of the mesencephalon increased. An extract containing the hypophyseal bromine was said to produce apathy when injected into dogs. The authors presumed that the pituitary secreted a substance containing bromine and that this substance could act upon the cerebral centres, facilitating the production of sleep.

The method of Roman [1929] and Pincussen [1932] employed by Zondek for these investigations has been studied by different authors [Fleischacker and Scheiderer, 1933; Hahn, 1933, 1; Holtz and Roggenbau, 1933] and has been shown to be subject to gross errors. Bier and Roman [1933] did not succeed in the quantitative determination of bromine solutions given to them by the above-named authors. The method on which Zondek based his conclusions seems therefore to be unsuitable for the estimation of bromine in blood and tissues.

Other methods have recently been described. Leipert and Watzlawek [1934] destroyed the organic matter by sulphate of silver and chromic and sulphuric acids in a special apparatus, collected the halogenous vapours in NaOH and, using the formation of hypochlorite to oxidise bromides to bromates, determined bromine by iodimetric titration. Dixon [1934; 1935] utilised the same reaction, destroying the organic substance by incineration with alkali. As Dixon pointed out, in this type of reaction there will always be formation of chlorate which will liberate iodine. The quantities of iodine liberated in "blank" estimations were equivalent to 0.4 ml. of *N*/500 thiosulphate (=10.7 γ of bromine). After ignition with alkali, Francis and Harvey [1933] liberated bromine by a chromic-phosphoric acid mixture, collected it directly in a KI solution and titrated with thiosulphate. Yates [1933] used a chromic-sulphuric acid mixture of a certain concentration which is said to oxidise bromides completely without oxidising chlorides.

As a specific reaction for the determination of bromine seemed to be more satisfactory than one based on the general principles of oxidation-reduction, the author studied the colorimetric bromine reactions of Guareschi [1913] and Baubigny [1897]. The former, used in Bernhardt and Ucko's investigations, was found to be less suitable for exact quantitative determinations than Baubigny's reaction. This reaction consists in the formation of tetrabromofluorescein (eosin) by the action of bromine on fluorescein. The method employed is as follows.

Method.

To 10 ml. oxalated blood (0.2 g. potassium oxalate per 100 ml. of blood) or an equivalent weight of triturated organs in a nickel crucible is added 0.25 g. pure NaOH per g. dry weight of the material. The mixture is evaporated to dryness on a water-bath and then heated on a wire-gauze over a small flame. After the swelling of the mass and the formation of tarry vapours have ceased, it is heated again over a flame at a gradually increasing temperature and kept at a dark red heat for 2-3 min. (observed in a dark room). After cooling, the ash is moistened with water to dissolve the pyrophosphates which inhibit the further ignition. It is then dried and heated in the same way and this procedure is repeated till almost white ash remains (in case of blood the ash is brick-red). In this manner the organic matter is completely destroyed at a low temperature in 7-8 hours. The ash is moistened with very small quantities of hot water, thoroughly stirred up with a glass rod over a small flame and the contents filtered into a 10 ml. flask. This process is repeated several times and, provided that these precautions are adopted, 10 ml. of water are sufficient to extract the bromides quantitatively. If a very low bromine concentration is expected, the liquid is filtered into a small Erlenmeyer flask and the volume reduced by heating to 3-5 ml. The filtrate is then neutralised in presence of a small piece of litmus paper with 25 % H_2SO_4 and 33 % NaOH. The solution is vigorously shaken after the addition of each drop to aid the elimination of CO_2 . The total amount of the liquid must be noted.

For the colorimetric reaction, 0.04-0.06 up to 0.26 ml. of a ten times diluted standard solution, containing 0.1489 g. KBr (=0.1 g. Br) per litre, are delivered into a series of test tubes of 5 ml. capacity and absolutely uniform diameter. The contents of the tubes, containing 0.4-0.6 up to 2.6 γ of bromine, are made up with water to 1 ml.

Preliminary investigations have shown that a well-defined colour is given with amounts as small as 0.1 γ Br, provided the reaction is adjusted to p_{H} 5.3-5.4. Therefore, 0.5 ml. of a buffer solution consisting of a mixture of 1 part of *N* acetic acid and 4 parts of *N* sodium acetate is added to each tube.

One drop of a 0.125 % solution of fluorescein is added to each tube. This solution is prepared with fluorescein twice recrystallised from alcohol, 1.2 g. being dissolved in 25 ml. *N* NaOH and made up with water to 1 litre. It can be kept in the dark for several weeks.

To oxidise the bromides to bromine, one drop of a 0.4 % solution of chloramine T is added. The use of chloramine instead of chlorine water or hypochlorite was suggested by Hahn [1933, 2].

The tubes are shaken at once and the reaction is stopped, after exactly 1 min., by addition of two drops of 20 % ammonia and one drop of sodium bisulphite. On examining the different pink colours on a white ground in the day-light, differences of 0.1 γ of bromine are easily recognised.

The reaction is carried out in the same way with different quantities of the tissue filtrate, the colours compared with those of the standard range and the

arithmetic mean noted. This comparison gives better results than the use of a colorimeter.

About 200 bromine estimations have been carried out by this method. The only objection is the time and care to be devoted to the destruction of the organic matter (which has to be watched carefully). A suitable method of wet ignition would be more satisfactory.

Blank determinations showed that the quantities of reagents and of doubly distilled water employed did not contain appreciable amounts of bromine so that special purification of the reagents was unnecessary. The colorimetric estimation in the blood filtrate itself, without isolation of the bromine compounds, has the further advantage of avoiding any loss of bromine. The addition of phosphates, sulphates, chlorides and carbonates to bromine solutions did not affect the colorimetric reaction.

The presence of chlorides does not affect the reaction. Iodine forms with fluorescein the similarly coloured tetraiodofluorescein. Normally, there is less than 0.1 γ iodine in the quantities used for one estimation. In the case of tissues rich in iodine the latter must be previously separated.

Control of the method.

(1) In about thirty estimations of aqueous bromide solutions containing different quantities of bromides not known to the operator (from 0.4 to 3.5 γ) the error was below 0.1 γ .

(2) After the addition of dibromotyrosine to an organic mixture, otherwise free from bromine, and ignition with alkali, bromine was found with an accuracy of 3–4 %.

(3) The differences in about a hundred duplicate estimations of organic material were less than 5 %.

(4) The following table shows the amounts of bromine found in human blood and urine together with amounts after bromide has been added in quantities not previously known to the operator.

Table I.

Material	Br without addition mg. per 100 ml.	Quantity of Br added mg. per 100 ml.	Total Br		Differences %
			Calculated mg. per 100 ml.	Found mg. per 100 ml.	
Blood D	0.189	0.150	0.339	0.334	- 1.5
" F	0.293	0.150	0.443	0.452	+ 2.0
" B	0.126	0.120	0.246	0.256	+ 4.0
" A	0.137	0.100	0.237	0.229	- 3.4
" Ph	0.171	0.180	0.351	0.336	- 4.3
" M	0.195	0.150	0.345	0.318	- 7.9
Urine U	0.441	0.600	1.041	1.040	- 0.1

According to the calculation of the probable error, the maximum error of the method would be 8–10 %. Practically, it was seldom higher than 5 %.

Results.

(1) In almost all *salts*, especially in halogen derivatives, bromine has been found. One sample of chloride of sodium, claimed to be chemically pure, contained 1.55 mg. of NaBr per 100 g.

(2) *The blood.* About 100 samples of human blood have been analysed. Bromine was regularly found. The figures, obtained from the blood of normal

persons never treated by bromine-containing medicaments, varied between 0.15 and 0.35 mg. per 100 ml. These values are lower than those previously recorded. Similar figures were found in the blood of dogs. The great variations in normal blood bromine recorded by some authors [Guillaumin, 1933; Yates and Hennelly, 1935] were not observed. As the normal chlorine and iodine concentrations in the blood are very constant, a relative invariability in the normal blood bromine seems to correspond well with the conditions of the other halogens. The ratio Br to Cl, *i.e.* Br/Cl in the blood was 0.0005–0.0014.

After bromine intake, the blood bromine rises markedly, while the total halogen content remains normal. As the rate of bromine elimination is very slow, an increased amount of bromine in blood and tissues can be found many months after the intake of Br has ceased. This fact may be one of the causes for the variable figures recorded by different authors. In France,¹ where these investigations have been performed, bromine-containing drugs are very rarely administered, and the author has had the opportunity of examining a large number of cases untreated by such medicaments [Ueko, 1934].

In ten cases the blood bromine level was high (0.6–3.0 mg. per 100 ml.). In two of these cases no evidence of previous bromine or iodine administration was found, and both suffered from Graves' disease: the blood bromine of one of them was 1.8 mg. per 100 ml. In neither case was iodine separated. Two other patients with Graves' disease showed normal figures.

The relative distribution of bromine between corpuscles and plasma was found to be very constant whether it was present in normal or artificially increased amounts. The ratio was about 1:3, lower therefore than that found for chlorine. No difference was found between the bromine content of whole and defibrinated blood, between plasma and serum and between corpuscles and blood-clot: from this it may be concluded that the Br content of plasma proteins is negligible.

To obtain some idea of the chemical state of bromine, the alcohol-soluble and insoluble fractions of bromine have been separated in two samples of normal blood, as has been done in the case of iodine [v. Fellenberg, 1926; Veil and Sturm, 1925].

To 1 part of total blood 4 parts of 95% alcohol are added, the precipitate is filtered by suction, washed thrice with alcohol, the alcohol is distilled off and the residue and the precipitate separately ignited and analysed. About 1/5 of the total bromine was found to be in the alcohol-insoluble part of the blood, which suggests that this fraction of the blood bromine is present in some organic complex.

(3) *The urine.* No record of systematic investigations into the normal bromine content of the urine has been found. In about seventy 24-hour specimens examined bromine was constantly present, the amounts eliminated varying from 1.0 to 2.5 mg. The bromine content varied very much in samples passed at different periods of the day, but these variations were always related to the amount of chlorine (Br/Cl=0.0003–0.0005) and to the specific gravity. The Br/Cl is therefore much lower in the urine than in blood and tissue (see below). After the intake of large amounts of bromine the bromine output is increased and the ratio rises. The highest ratio found among the cases examined was 0.1–1. An increased urinary bromine was always associated with a high blood bromine level.

In cases where retention of bromine was very marked, the Br/Cl was found to be very similar in urine and blood.

¹ Hôtel-Dieu, Paris (Prof. Carnot).

In one determination of the alcohol-insoluble part of the urinary bromine, only traces were found suggesting that the urinary bromine was entirely present in an alcohol-soluble form.

After intravenous injection of 1 g. NaBr (=780 mg. bromine) the average bromine elimination was:

	mg. Br
1- 4 hours after injection	7-11
4- 8 " "	2-3
8-24 " "	5-10
Total amount in 24 hours	14-24

The elimination of very small doses (0.3-0.5 mg. daily) was studied in a dog. The animal was fed on a standard diet on which the daily urinary output contained 1.2-1.4 mg. Br. After 8 days of this diet small doses of bromine were given daily with a total of 15 mg. in 35 days. The output of urinary bromine increased in the first 2 days to a figure of 1.7-1.9 mg. and remained at that level for the rest of the experimental period. At the end of the experiment the blood bromine had risen from 0.180 to 0.286 mg. per 100 ml. indicating an increase of about 0.63 mg. of bromine in the blood (=1/13 of body weight). In proportion to the intake much more bromine was eliminated during 24 hours in this experiment than after the injection of one large dose.

(4) *The gastric juice.* In ten normal patients 0.5-0.9 mg. per 100 ml. (in one case 1.2 mg. per 100 ml.) of bromine were found in the gastric juice of the "fasting" stomach. It seems to be present "normally" and in a higher percentage than in the blood. The Br/Cl was between 0.0017 and 0.0055.

The effect of histamine upon the bromine content of gastric juice was studied. After withdrawal of the fasting juice 1 ml. of 1% histamine phosphate was injected and gastric samples collected at 10-min. intervals. The bromine, total chlorine and free hydrochloric acid were estimated in each sample and the Br to Cl ratio calculated. Table II gives the results of three typical experiments. It will be seen that histamine has no apparent effect upon the normal bromine content, despite great variation in the chlorine content and free acidity.

Table II.

Time in min. after injection	Bromine mg. per 100 ml.			Chlorine mg. per 100 ml.			Free acid = mg. per 100 ml. (%)			Br/Cl		
	1	2	3	1	2	3	1	2	3	1	2	3
Initial	1.2	0.76	0.57	308	292	346	102	73	73	0.0055	0.0026	0.0017
10	1.19	0.80	0.58	481	407	316	314	230	175	0.0025	0.0020	0.0018
20	1.15			537			387			0.0021		
30	0.87			505			226			0.0017		
40	0.73			423			153			0.0017		

The effect of intravenous injection of bromide was studied in a similar manner. 1 g. NaBr was injected intravenously and gastric samples were removed

Table III.

Time in min. after NaBr	mg. per 100 ml. Br after NaBr injection
Initial	0.82
10	10.0
20	12.0
30	13.0
40	—
50	13.0
60	

at 10-min. intervals. Table III gives the result of a typical experiment and it will be seen that there is a marked and maintained rise in the bromine content. The effect of histamine upon this raised output was investigated (Table IV) and no appreciable difference was recorded.

Table IV.

Time in min. after NaBr	Time in min. after histamine	mg. per 100 ml. Br after NaBr and histamine injection	Br/Cl
Initial	—	0.87	0.0024
10	—	—	—
20	—	—	—
30	10	13.4	0.0355
40	20	13.7	0.0299
50	30	13.2	0.0287
60	40	14.1	0.0310

If repeated doses of bromide have been taken so that a large part of the gastric chlorine has been replaced by bromine, histamine seems to have an effect on the gastric bromine level so that, under these conditions, bromine appears to have become an integral component of the secretory response. An example of this is given in Table V.

Table V. *Histamine effect after intake of 6 g. NaBr by mouth daily during 5 days (total amount = 28 g. bromine).*

Time in min. after histamine	Bromine mg. per 100 ml.	Total halogens calculated as mg. per 100 ml. Cl	Chlorine mg. per 100 ml.	Free acid - mg. per 100 ml. Cl
Initial	108	284	236	18
10	160	500	430	280
20	143	572	520	419
30	130	550	492	406
40	135	547	487	391
50	160	543	468	351

(5) *The organs.* Table VI shows the bromine figures obtained by the analysis of different organs.

Unfortunately, human pituitaries could not be obtained for analysis. In animals (except in the case of one bull) the hypophyseal figures were not higher than those of other organs. Dixon found the bromine content of human pituitaries to vary from 0.4 to 2.4 mg. per 100 g., calculated for wet weight; in four exceptional cases it was 4.4, 4.0, 9.0 and 26. The last two cases had previously received bromine and in the first cases some possibility of bromine dosage was presumed.

Serbescu and Buttu [1934] claimed recently that the thyroid gland contains 2-3.1 mg. per 100 g. of bromine although the method they employed failed to demonstrate the presence of bromine in the blood and in most human organs (limit of sensitivity of method 30 γ). The authors have apparently not succeeded in removing all iodine from the thyroid, a necessary preliminary for the determination of bromine by colorimetric methods.

A satisfactory procedure for the removal of iodine is to oxidise the iodides in an acid medium by the addition of a few drops of a 10% solution of sodium nitrite. Iodine is collected by shaking in chloroform. Nitrous acid does not oxidise bromides if present in a low concentration. The process is repeated till

Table VI.

Organ	Species	Bromine mg. per 100 g.	Chlorine mg. per 100 g.	Br/Cl
Pituitary	Ox	0.59	206	0.0029
"	Ox	0.78	—	—
"	Ox	0.59	—	—
"	Cow	0.61	168	0.0037
"	Cow	0.55	178	0.0031
"	Bull	1.47	182	0.0080
Pituitary, commerc. powder		3.3 = 0.66*	1170	0.0028
Suprarenal	Ox	0.39	—	—
"	Ox	0.58	144	0.0040
"	Cow	0.62	144	0.0043
Suprarenal, commerc. powder		4.9 = 1.2*	586	0.0086
Suprarenal	Man	0.66	171	0.0038
"	Man	0.33	180	0.0018
Testicle	Ram	0.57	175	0.0033
Epididymis	Ram	0.80	—	—
Ovary	Calf	0.55	151	0.0037
Ovary (two corp. lut.)	Cow	0.61	—	—
Pancreas	Dog	0.10	—	—
"	Dog	0.13	—	—
Thyroid	Ox	0.89	123	0.0073
"	Cow	0.75	112	0.0067
"	Dog	0.42	115	0.0037
"	Dog	0.94	—	—
"	Man	0.48	—	—
Thyroid, commerc. powder		5.6 = 1.1*	—	—
Spleen	Dog	0.26	—	—
"	Man	0.33	200	0.0017
"	Man	0.24	158	0.0015
Liver	Man	0.17	—	—
Liver (cirrhosis)	Man	0.14	—	—
Kidney	Man	0.27	165	0.0016

* Wet (calculated).

the chloroform gives no coloration with a starch solution. Using these precautions the bromine content of the thyroid (see Table VI) was found to be not markedly higher than that of other organs.

(6) *Bromine in mental diseases.* Ten typical manic-depressives were examined and the amounts of bromine in blood and urine determined during the active phases of their mental disease. In nine of them the figures for blood and urinary bromine lay within normal limits. The tenth patient had been treated previously with bromides and had a blood bromide of 26 mg. per 100 ml. (Br/Cl=0.096) and a urinary excretion of 19 mg. Br in morning sample (Br/Cl=0.08). Two cases, one immediately after an epileptic fit and one in a state of stupor, showed similar normal bromine figures. Though the number of cases is small, it can be concluded that there is no constant diminution of blood bromine in cases of manic-depressive psychosis.

CONCLUSIONS.

As bromine is eliminated extremely slowly from the body and can accumulate in fluids and tissues in very great amounts, "normal" figures can be misleading and high figures, *e.g.* pituitary, may be due to unrecorded previous administration of bromine-containing drugs. Bernhardt and Ucko [1926] pointed out that it was unlikely that the inorganic bromine of the body had any specific functional significance owing to the fact that it accumulated so easily in the body without

producing any obvious effect. If this assumption is correct, bromine could only have a specific rôle as a normal constituent of the body if it were present in a special chemical state. The observation that 1/5 of the blood bromine is found in the alcohol-insoluble part of the blood, but only traces in the alcohol-insoluble part of the urine, suggests the possibility of the existence of some bromine complex which is not allowed to escape *via* the kidney.

The main source of bromine is the food, especially common salt. Plants regularly contain small quantities of bromine [Damiens and Blaignan, 1931; 1932]. The "normal" figures will therefore only refer to the bromine content on a "normal" diet. The maintenance of a constant level of blood chloride despite great variation in chlorine intake points to a delicate regulation of the chlorine balance in the body: a similar type of regulation would appear to control the bromine level. This regulation is sufficiently delicate to prevent any accumulation of bromine as a result of dietetic variations or following very small doses of bromides. It would appear that the smaller the dose of bromine taken, the smaller is the proportion retained. This may explain why the food bromine does not accumulate in the body. After the administration of large doses, bromine seems to be stored in tissues and body fluids, as previous observations and its appearance in the gastric juice suggest.

Normally, a definite relationship exists between the distribution of bromine and chlorine in the body. The ratio Br to Cl is 5-10 times smaller in the urine than in the tissues.

	Br/Cl (average)
Organs	0.0015-0.0080
Gastric juice	0.0017-0.0055
Blood	0.0005-0.0014
Urine	0.0003-0.0006

An explanation of the low bromine content of the urine would be that normally the kidneys do not excrete bromine above a certain limit. It is therefore retained in the body in higher proportions when present in large amounts. Viewed in this light, the replacement of chlorides in the body by bromides, formerly attributed to a "lack of discrimination" on the part of the kidneys regarding halogens, would be due to a restriction of bromide excretion and to a compensatory increased excretion of chlorides to maintain the normal osmotic equilibrium. Thus the total halogen level in the body remains unaltered.

The kidneys, however, are capable of excreting far larger amounts of bromine than they do normally. If a certain degree of bromine storage has been established, we see that even small quantities of urine may contain several mg. of bromine. Under these conditions, the Br to Cl ratio rises till it becomes of the same order as that of the blood, so that bromine is then excreted in the same proportion to chlorine as it exists in the body.

We do not know whether the physiological retention of bromine depends solely on a renal factor or whether a tissue factor is also concerned. Nor do we know whether the high output after bromine storage is due to an increased permeability of the normal renal filter or to a lack of storage capacity in the tissues.

SUMMARY.

1. A method is described and criticised for the determination of bromine in body fluids and tissues.

2. The figures for bromine found in blood, urine, gastric juice and tissues in normal cases and after the intake of bromine are recorded.

3. Ten manic-depressive patients showed normal figures both in blood and urine.

4. The bromine equilibrium in the body and its relationship to the chlorine content are discussed.

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CXLIV. ENZYME FORMATION AND POLYSACCHARIDE SYNTHESIS BY BACTERIA. II.

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AN account was given in a previous communication [Cooper and Preston, 1935] of the study of the biochemical conditions influencing the formation and activity of diastase and invertase in bacteria, and also of the synthesising enzyme associated with the formation of polysaccharides of the "laevan" type by bacterial action. It was suggested in that paper that the investigations would be of wider physiological interest if they could be extended to the formation of other polysaccharides such as dextrans, galactans, pentosans, in addition to laevans. Experiments in this direction were commenced with numerous micro-organisms, but in the case of the aerobic sporing bacilli and the plant-pathogenic bacteria their synthetic capacity was limited to "laevan formation".

Further investigations with other micro-organisms have now been carried out and are described in the present paper.

The general experimental methods and basal culture media employed were the same as those used in the previous work, the principal culture medium being a solution of

	%
Peptone	0.1
Na ₂ HPO ₄ . 12H ₂ O	0.2
KCl	0.5
Carbohydrate	10

SECTION I.

The micro-organisms tabulated in Table I were cultivated in 10 or 20 ml. portions of the foregoing medium containing 10% of various carbohydrates. The cultures were incubated at the optimum temperature of the particular organism for 7-10 days, and evidence of polysaccharide synthesis was looked for by filtering the cultures and allowing the filtrate to run into three times the volume of alcohol with mechanical stirring. The polysaccharide was thrown out as a white precipitate and purified by redissolving in water and precipitating twice with alcohol, grinding with alcohol and then ether and finally drying in a vacuum desiccator.

Table I.

Micro-organism	Polysaccharide formation
<i>Bacillus lactis</i>	"Fructosan" from sucrose only
<i>Bact. fluorescens liquefaciens</i> ¹	Nil
<i>Bact. acidi lactici</i>	
<i>Bact. hyacinthi</i>	Trace from sucrose
<i>Chromobacter viscosum</i>	Trace from sucrose and galactose
<i>Micrococcus lactis viscosus</i>	Nil

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Bacillus lactis, occurring in milk, readily formed a polysaccharide from sucrose. This was laevorotatory and was readily hydrolysed by acids, yielding a sugar, which was also laevorotatory and formed glucosazone. The product thus resembled the laevans synthesised by other sporing bacilli and plant pathogens.

Bact. hyacinthi only produced a trace of polysaccharide from sucrose (5%), and a chemical examination was not possible. The organism failed to grow in 10% sucrose solution.

Chromobacter viscosum. According to Harrison and Barlow [1905] this organism produces a slime from sucrose, lactose and galactose. We have confirmed these observations but have not been able to isolate the polysaccharide, owing to the small amount synthesised by the particular culture. Replacement of peptone by asparagine, glycine, alanine or leucine did not increase the yield, although the organism grew satisfactorily. There was no growth, however, with succinamide, malonamide, urea and methylurea as sources of nitrogen.

In the case of this organism, it has not been possible to stimulate enzyme-formation or to increase the amount of polysaccharide synthesised.

SECTION II.

Polysaccharide formation by Leuconostoc species.

Dextran formation by *Leuconostoc* was investigated by several earlier workers, especially by Liesenberg and Zopf [1892], Zettnow [1907] and Beijerinck [1912]. Tarr and Hibbert [1931], however, were the first to investigate accurately the conditions of formation of dextran by *Leuconostoc mesenteroides*, and these workers have provided an extensive bibliography. They found that the polysaccharide is synthesised by this organism in a peptone-phosphate medium from sucrose, but only in a very small amount from glucose and not at all from other carbohydrates. In the case of sucrose dextran production increased with rise in concentration up to 20% and attained a maximum within a period of 10 days. Tarr and Hibbert were able to prepare large amounts of the dextran for chemical investigation.

We have continued these investigations with *L. mesenteroides*, the organism being grown at 30° in the same culture medium as described in the previous section. With two cultures the yield of "dextran" was very small indeed. We endeavoured to increase polysaccharide formation by replacing or supplementing the peptone by 0.1% concentrations of various amino-acids (glycine, *d*-alanine, asparagine, *l*-leucine, glutamic acid). Only in the case of *l*-leucine was any difference noted, a perceptible increase in polysaccharide production occasionally being observed, but the improvement was not sufficiently marked for a large-scale synthesis. Subsequently we attempted to isolate active organisms from "leuconostoc slime". The inoculation of the 10% sucrose culture medium with a particle of the slime rapidly caused opalescence after only 48 hours' incubation at 30°, and by precipitation with alcohol a dextrorotatory polysaccharide was obtained, yielding glucose after hydrolysis with acid. A culture was therefore plated out on sucrose-nutrient agar, and pure cultures were isolated and maintained on the same medium. None of these cultures, however, produced polysaccharide in a sucrose-peptone medium, notwithstanding the synthetic activity of the organisms in the original slime. Moreover, the capacity of the mixed slime organisms was lost on repeated subculture into fresh sucrose-peptone medium. We then found that Hucker and Pederson [1930; 1931] had stated that most *Leuconostoc* strains require an accessory growth substance, present in yeast or tomato extract, in addition to the ordinary peptone-nitrogen supply. Allison

and Hoover [1934] and Thorne and Walker [1934] also showed that certain nutritive factors present in yeast, molasses and egg albumin were essential for the growth of *Bact. radicum*, and our experience in this laboratory with this organism has fully confirmed their conclusions.

We therefore decided to investigate the influence of the presence of raw beet-sugar and molasses on polysaccharide synthesis by *Leuconostoc*, and samples for the purpose were kindly supplied by the West Midland Sugar Co., Ltd.

The cultivation of the foregoing organisms (isolated on the nutrient agar medium) in peptone-sucrose solutions, containing varying proportions of raw beet-sugar or molasses, however, did not lead to any improvement, as polysaccharide was still not produced. The organisms present in the original crude slime, on the other hand, maintained their dextran-producing capacity with repeated subcultivation in raw sugar media, although, as already pointed out, this was rapidly lost in pure sucrose cultures.

The technique of isolation of the organisms from the crude slime was, therefore, repeated, employing nutrient agar, containing 8% sucrose and 2% molasses, and the pure cultures were maintained on this medium (with the addition of calcium carbonate) throughout the experimental work. Certain of the colonies isolated during the process of plating out were now vigorously gum-producing and became surrounded by a highly viscous secretion. Four types of organism were finally selected and stock cultures kept on the molasses medium. The capacity of these cultures to synthesise dextran was tested in (a) 10% sucrose, (b) 6% sucrose + 4% raw beet-sugar, (c) 8% sucrose + 2% molasses, peptone being employed as source of nitrogen. The synthetic powers of two identified varieties of *Leuconostoc* kindly sent to us by Prof. A. J. Kluyver¹ were also compared in the same media.

The results are set out in Table II.

Table II.

Organism		Dextran-formation (6 days' incubation at 30°)		
		(a) 10% Sucrose	(b) 4% Raw beet- sugar + 6% sucrose	(c) 2% Molasses + 8% sucrose
Organisms isolated from the slime	A	—	++	++
	B	—	—	—
	C	—	—	—
	D	+	++	+++
<i>Betacoccus arabinosuccus anhaemolyticus</i> (Kluyver) (<i>Leuconostoc mesenteroides</i>)		+	++	++
<i>Betacoccus arabinosuccus haemolyticus</i> (Kluyver) (<i>Leuconostoc dextranicum</i>)		++	+++	+++
— No dextran formation.		+ Slight.	++ Moderate.	+++ Maximum.

The results indicate that in the case of all synthesising cultures, dextran formation was greatly increased by the presence of raw beet-sugar or molasses. Only one organism (*B. arab. haemolyticus*) produced dextran actively in pure sucrose medium, and even this was stimulated by raw sugar: organism A, which produced great viscosity on molasses-agar, not only failed to synthesise dextran from pure sucrose but also failed to grow in this medium. Two organisms B and C did not possess any synthetic capacity in any of the three media employed.

B. arab. haemolyticus (*L. dextranicum*) produced great viscosity in sucrose solutions, with or without raw sugar or molasses, the viscosity being so marked

¹ These varieties of *Leuconostoc* are classified under the generic name *Betacoccus* (see Table II).

that the cultures could be safely inverted. In pure sucrose medium viscosity was well marked within 4 days; in the presence of raw sugar or molasses it was apparent in 2 days and sometimes in 24 hours. Notwithstanding this initial stimulation, it is important to note that after 10 days' incubation the yield of polysaccharide (by weight) was practically the same.

Proof that the polysaccharides synthesised by the foregoing organisms were of a dextran nature was afforded by the following evidence. The polysaccharides were precipitated by the addition of alcohol (equal volume) to the viscous cultures and purified by the method already described in Section I. The products from all these organisms were entirely soluble in hot water, giving dextro-rotatory solutions. After prolonged hydrolysis with $N H_2SO_4$ they yielded a sugar, which had the specific rotation (approximately) of glucose and formed glucosazone with phenylhydrazine.

On account of the readiness with which *L. dextranicum* synthesises a dextran from sucrose with the production of an easily-recognised viscosity, we have employed this organism in some further researches, with the object of throwing light on the two following problems:

(a) An explanation of the failure of this organism to produce dextran from glucose.

(b) The chemical nature and rôle of the so-called accessory growth factor present in raw beet-sugar and molasses.

(a) *Action on glucose.* The inability of this organism to synthesise dextran from 10 % glucose solution except in very small amount was first confirmed. It was observed that growth was poor and viscosity did not develop, and on adding alcohol to the glucose cultures only a turbidity or very small amount of precipitate was obtained. Identical results were obtained when very small amounts of beet-sugar or molasses were added to the glucose medium to stimulate the organism, and there was no improvement in yield by working at concentrations of glucose of 2 or 5 %.

This failure to produce dextran was not apparently due to the inhibitory effect of acid formation from the glucose, as the p_H of glucose and sucrose cultures after a week's incubation was of very much the same order (about 4).

From these results it might be provisionally inferred that the dextran is synthesised from the fructofuranose section of the sucrose molecule.

We attempted to test this possibility by studying the effect of *L. dextranicum* on laevan synthesised by *B. pruni*. The laevan, however, was not attacked, and there was no evidence of dextran-formation.

An examination of the reducing sugars present in cultures of *L. dextranicum* in 10 % sucrose media was therefore next undertaken. It was found that after removal of the polysaccharide by alcohol the solutions were definitely laevo-rotatory, and from the optical activity and reducing power it was calculated that the reducing sugar present was mainly fructose, suggesting that the dextran had been built up from the glucose. Notwithstanding, a rôle might still have to be ascribed to fructofuranose in order to account for the dextran formation from sucrose only, and it seemed possible that, whilst the dextran is actually synthesised from the liberated glucose, yet the nascent fructofuranose might be essential in the constructive metabolism of the organism. However, in experiments in which small amounts of sucrose were added to glucose there was no utilisation of glucose for polysaccharide formation. On the contrary, the yield of dextran from sucrose was perceptibly diminished in the presence of excess of glucose.

At the present time no explanation can be offered to account for the slight growth and low synthesising activity of the organism in glucose media, compared with sucrose.

Experiments have also been carried out to ascertain whether there is evidence of the formation of a synthetical enzyme in a peptone medium containing 2% glucose and also 1% molasses. The organism was grown in this medium (10 ml.) for 3 days; at this stage toluene was added and also 10 ml. of 20% sucrose. Incubation was continued at 30° for a week, and the culture was then poured into three times its volume of alcohol. No precipitate was obtained, indicating that dextran had not been formed, and therefore an active synthesising enzyme had not been produced in the original culture.

The question as to whether *L. dextranicum* could utilise as a carbohydrate source the dextran it synthesised was next examined. The organism was sub-cultured into a peptone-phosphate medium, containing 1% dextran, and after 10 days the medium was tested for reducing sugar and change in p_{H} . No evidence of hydrolysis of the polysaccharide or production of acid was obtained, showing that the organism had not utilised the synthesised dextran as a reserve carbon supply under the conditions employed.

The results are analogous with those obtained by Anderson [1933] who found that the gum synthesised by *Rhizobium* was not utilised by the organism. In both cases polysaccharide synthesis appears to be an irreversible process.

(b) *The stimulating action of raw beet-sugar and molasses on polysaccharide formation.* In the first place experiments were carried out to ascertain the minimum amount of these substances essential to effect a definite stimulation. Varying amounts of raw beet-sugar or molasses were therefore added to sucrose-peptone cultures, the amount of pure sucrose being adjusted to maintain the sugar concentration at 10%. It was found that comparatively small amounts were adequate to accelerate the development of viscosity. 0.2% of either raw beet-sugar or molasses produced viscosity in 24 hours, whereas in this experiment the sucrose-peptone control culture required 96 hours. However, we also found that 0.5% of molasses could replace the peptone, viscosity being obtained in 24 hours. Therefore, the presence of available nitrogen in the molasses had to be considered.

When the peptone was replaced by 0.1% of any of the following nitrogen compounds: glycine, *D*-alanine, *L*-leucine, asparagine, glutamic acid (sodium salt), creatine, malonamide, succinamide, ammonium succinate, urea or methyl-urea, no growth or dextran formation took place in the absence of raw sugar or molasses, except on one occasion with *D*-alanine, when slight viscosity developed. The addition of small amounts of histidine, lysine or proline to supplement the foregoing nitrogen compounds or peptone was also without effect.

These results point to the conclusion that the essential substance present in beet-sugar and molasses cannot be identified with any of these amino-acids or amides.

The fact just mentioned that viscosity is produced in peptone-sucrose medium, but not in media in which the peptone is replaced by pure amino-acids and amides, suggested, however, that peptone contains a certain proportion of the substance present in raw sugar and molasses, essential for growth and dextran synthesis. The requisite structure could, of course, be either in the combined or free state in the peptone.

50 g. of peptone were therefore refluxed with 200 ml. of absolute alcohol for 24 hours, the mixture filtered and the filtrate concentrated *in vacuo* and finally made up with distilled water to 20 ml. It was sterilised before use. The

extracted peptone residue was again refluxed with alcohol, washed with alcohol and ether and dried in the steam-oven. Culture media containing 0.1% of the original and the extracted peptone with 10% sucrose and the usual salts were then prepared for comparison.

It was found (see Table III) that whilst the viscosity developed in the presence of the original peptone in 48 hours, with the extracted peptone it was delayed until 72 or 96 hours, and sometimes had not developed in 2 weeks. The addition of 0.05 or 0.1 ml. of the peptone extract to either original or extracted peptone on the other hand accelerated the development of viscosity.

The substitution of 0.05 ml. of the peptone extract for peptone as sole source of nitrogen also readily produced viscosity in 48 hours.

0.01 ml., however, contained a marginal amount of available nitrogen for the organism, as viscosity was postponed until the 10th day of incubation. The percentage of nitrogen in this extract was 0.375. It is interesting to record, however, that the addition of this limiting dose (0.01 ml.) of extract to culture media containing the extracted peptone did not accelerate the appearance of viscosity, and a similar addition to media containing 0.1% asparagine, alanine, or ammonium succinate did not result in the development of viscosity any earlier than in the control experiment (10 days). Thus, when the amount of extract is so reduced, in order to restrict the available nitrogen, there is no evidence at all of any stimulation. We have been able, however, to demonstrate stimulation by using extracts of molasses under conditions where available nitrogen, if present, was inadequate. These extracts were prepared as follows. 100 g. of molasses were refluxed with alcohol for 6 hours, the mixture being shaken occasionally during this period. The clear alcoholic layer was poured off and the extraction of the molasses was repeated during 3 hours with 100 ml. of alcohol. The combined alcoholic extracts were evaporated under diminished pressure to a syrup which was dissolved in about 50 ml. of hot absolute alcohol and allowed to stand overnight at a temperature of 5-10°. This cooling caused the precipitation of sucrose and on pouring off the alcoholic solution and evaporating *in vacuo* a small amount of a brownish yellow residue was obtained. This residue was then extracted with 100 ml. of ether, the ether extract poured off and the ether-insoluble portion extracted with 100 ml. of alcohol-ether (50:50). The alcohol-ether extract was poured off and the insoluble portion was dissolved with 100 ml. of absolute alcohol. The three solutions thus obtained were evaporated *in vacuo* and each of the residues was taken up in water. The ether-soluble portion was made up to 10 ml., the ether-alcohol portion to 20 ml. and the alcohol-soluble residue to 20 ml. These solutions are referred to as extracts A, B and C respectively in Table III.

It was found that these extracts, unlike the peptone extracts, were deficient in available nitrogen, even 0.5 ml. per 10 ml. of culture medium being inadequate as sole source of nitrogen for sustaining growth and polysaccharide synthesis. On the other hand, very small amounts, *e.g.* 0.01 ml., accelerated in a remarkable degree the development of viscosity in the media containing peptone or extracted peptone.

There was evidence, moreover, that extract C (alcoholic) was more efficacious than the alcohol-ether extract B. The ether extract A contained the stimulating factor, but we do not know yet whether the content is less than in the case of B or C.

The results indicate that the stimulating action is not due to the nutritive effect of the presence of available nitrogen in the extracts, but is dependent on a distinct factor, which is being further investigated.

Table III. *Influence of peptone and molasses extracts on dextran formation in 10% sucrose-phosphate. Total volume 10 ml.*

Nitrogen compound 0.1 %		Peptone ^a extract added ml.	Period required for development of viscosity (days)	Nitrogen compound 0.1 %	Molasses extract added ml.	Period required for development of viscosity (days)	Nitrogen compound 0.1 %	Molasses extract added ml.	Period required for development of viscosity (days)
Peptone	—	—	2	Peptone	—	3	—	A 0.5 B 0.5 C 0.5	No viscosity in 10 days
Extracted peptone	—	—	4	"	A 0.1	2	—	—	—
"	"	0.05	3	"	B 0.1	2	—	—	—
"	"	0.01	4	"	C 0.1	1	Ammonium succinate	—	—
—	—	0.05	2	"	0.05	1	"	—	—
—	—	0.01	10	"	0.02	2	d-Alanine	B 0.5	—
Glycine	—	—	—	"	0.01	2	"	C 0.5	—
d-Alanine	—	—	—	Extracted peptone	—	No viscosity in 10 days	Valine	C 0.1	—
L-Leucine	—	—	—	"	B 0.1	1	"	—	—
Asparagine	—	—	—	"	0.05	2	L-Leucine	—	—
Glutamic acid (Na salt)	—	—	No viscosity in 10 days	"	0.02	3	"	B 0.5	No viscosity in 10 days
Succinamide	—	—	—	"	C 0.1	1	(Glutamic acid (Na salt)	—	—
Ammonium succinate	—	—	—	"	0.05	1	"	C 0.1	—
Creatine	—	—	—	"	0.02	2	L-Histidine HCl	—	—
Urea	—	—	—	"	0.01	3	"	C 0.1	—
Methylurea	—	—	—	"	B 0.1	3	Asparagine	—	—
Asparagine	0.01	10	—	"	0.5	3	"	A 0.1	—
d-Alanine	0.01	10	—	"	C 0.1	1	"	B 0.1	—
Ammonium succinate	0.01	10	—	"	0.5	1	"	C 0.1	—
				"	0.5	1	"	C 0.5	Slight viscosity in 7 days

When the peptone was replaced by ammonium succinate and certain amino-acids (Table III) dextran formation did not take place, even in the presence of one of the stimulating extracts. In the case of asparagine with an excess of extract C, a slight viscosity developed after some delay (7 days). *Leuconostoc* is, therefore, much more specific in regard to its nitrogen requirements than the "laevan"-forming organisms, which synthesised the polysaccharide in certain amino-acid media as effectively as in the presence of peptone [Cooper and Preston, 1935].

Large-scale preparation of the dextran synthesised by Betacoccus arabinosaceus haemolyticus.

Equal volumes of sterile 20% sucrose solution and double strength peptone-salt medium (0.2% peptone) were distributed while hot into large Erlenmeyer flasks, the total volume of medium per flask being 800 ml. 5 ml. of sterile 50% molasses solution were added to all the flasks, which were then inoculated with a 48-hour culture of the organism in the same medium and incubated at 30° for 2 weeks. It was found advisable to avoid steaming the sugar and peptone solutions after admixture, as previous experience had shown that even in the presence of molasses the organism then occasionally failed to grow. The total volume of medium employed for the dextran synthesis was 5 litres.

After 2 weeks the contents of the majority of the flasks were highly viscous, and they were then poured into an equal volume of ethyl alcohol with vigorous stirring. The yield of crude dry polysaccharide was 121 g. (about 25% of the saccharose employed).

SUMMARY.

1. *Bacillus lactis* synthesises a polysaccharide from sucrose, but not from other carbohydrates. The product proved to be of the "fructosan" type, analogous to the "laevans" synthesised by other aerobic sporing bacilli and also plant-pathogens.

2. Dextran formation from sucrose by *Leuconostoc* species is greatly stimulated by the presence of raw beet-sugar and molasses.

3. There is evidence in the case of *L. dextranicum* (*Betacoccus arabinosaceus haemolyticus*) that the dextran is synthesised from the glucose portion of the sucrose molecule. Notwithstanding, even under varied conditions, the polysaccharide is only formed in very slight amount from free glucose.

4. Small amounts of raw beet-sugar, molasses or alcoholic extracts of peptone can supply the required available nitrogen for normal dextran synthesis, and can thus replace ordinary peptone in the culture media.

5. Alcoholic extracts of molasses, however, are deficient in available nitrogen, but minute proportions greatly accelerate growth and dextran formation in either peptone or alcohol-extracted peptone media. The stimulating action is thus dependent on some other factor than nitrogen supply.

6. Polysaccharide formation does not take place in media in which peptone has been replaced by certain amino-acids or ammonium succinate (Table III).

7. A method is described for the large-scale preparation of the dextran synthesised by *L. dextranicum*.

This investigation has been carried out during the tenure of a Leverhulme Research Fellowship by one of us (E. A. C.).

The authors desire to express their thanks to Prof. W. N. Haworth and Dr E. L. Hirst for their interest and encouragement; and to Dr R. St John Brooks, Dr G. J. Hucker, and Prof. A. J. Kluyver for kindly supplying the micro-organisms used in this research.

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CXLV. THE NITROGEN METABOLISM OF THE PEA SEEDLING.

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THE investigation of the lupin [McKie, 1931], an asparagine-producing plant, has been followed by that of the pea, in which no asparagine or other amide is formed in any amount, with a view to elucidating still further the part played by the various nitrogen metabolites. Similar conditions have been observed, and at the same time a parallel investigation of certain aspects of etiolated pea seedlings has been made.

•EXPERIMENTAL.

Pea seeds (*Pisum sativum*), about 6-12 per pot, were grown in batches of 50 in a good potting mixture from January to April, 1935. The temperature of the greenhouse varied from 50 to 67° F. For no batch were conditions extreme or in any way adverse. The harvesting and preparation of material for analysis were carried out as for the lupin [McKie, 1931].

Drying. The temperature recommended by Link and Schulz [1924] was finally adopted. The seedlings, from spirit, were dried at 65° for 3 hours, then ground to a fine powder in a mill and the meal thinly spread on filter-paper and dried in an air incubator at 65° for 48 hours. Analyses for total N (Kjeldahl), were made on this dried material.

Extraction of material. The dried and weighed material (6-7 g.) was intimately ground for 20 min. with 50 ml. of ammonia-free water and filtered through fine (mesh 0.017 cm.) weighed and dried muslin. This process was repeated, 6 times in all, using 30 ml. of water for each subsequent extraction, each muslin being pressed as dry as possible. The residue was dried to constant weight at 105°. Analyses were carried out as follows:

Residue. Total-N was estimated by Kjeldahl and expressed as "Insoluble N".

Filtrate. The combined washings were made up to 500 ml. (250 ml. for 0 (*a*) and 3 (*a* and *b*) days and 1000 ml. for 32 days), with ammonia-free water and analysed for total N and individual water-soluble N compounds. Analyses for total N, proteose, ammonia, amide and nitrate were performed as described for the lupin.

Protein. Owing to the presence in the pea of legumin, a protein soluble in acidulated water and incompletely coagulated by heat, a modified method of precipitation and filtration based on a study of the optimum conditions was adopted. The p_H of the extract was adjusted to 4.5, the isoelectric point of legumin, by addition of acetic acid, and the extract gently boiled for 1 hour. The coagulum was filtered hot through hardened filter-paper (Whatman No. 50) under slight pressure, repeatedly washed with hot water and dried to constant weight at 105°. Analyses for total N were then made on this material.

Proteose. Since some legumin inevitably remains after coagulation, and since this residuum is precipitated by Na_2SO_4 , the estimation of proteose by the method of Wasteney and Borsook [1917] gives proteose *plus* this residual protein. 5 ml. of extract were used for each determination.

Amino-acid. The Van Slyke method was adopted, 3 ml. of extract being used for each determination.

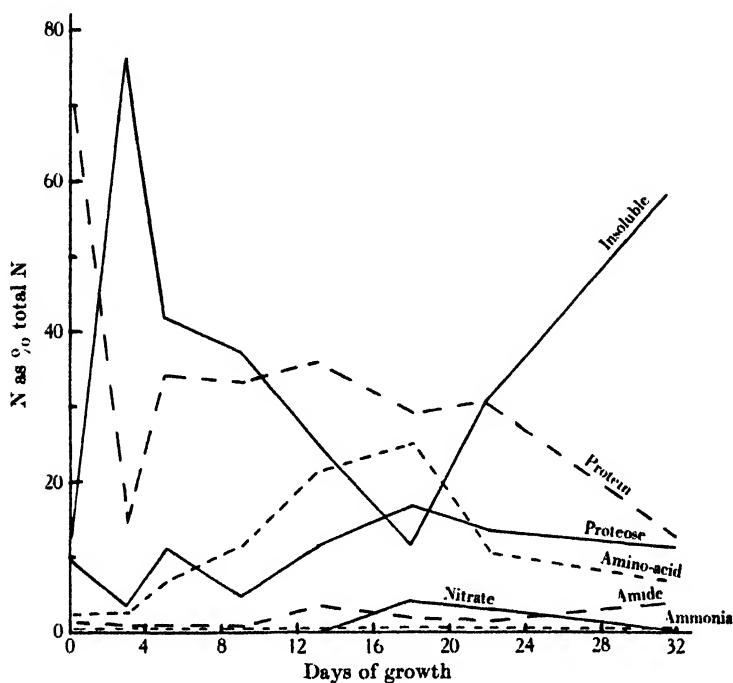


Fig. 1. Distribution of various forms of nitrogen.

Table I. *Variation of nitrogenous constituents.*

Age days	Seedling		Insoluble material			Coagulable protein		
	Dry wt. g.	Total N g.	g.	N %	N % dry wt.	g.	N %	N % dry wt.
0 (a)	12.11	0.48	3.79	1.46	0.46	4.40	7.90	2.87
(b)	12.23	0.49	3.59	1.41	0.42	5.02	7.32	3.09
3 (a)	6.05	0.25	4.26	4.29	3.03	0.80	4.98	0.66
(b)	8.76	0.36	6.35	4.58	3.33	0.97	5.00	0.55
5	5.01	0.24	2.57	3.86	1.98	1.44	5.69	1.64
9	7.20	0.38	3.08	4.74	2.03	2.05	6.33	1.80
13	4.65	0.26	1.50	4.55	1.47	1.49	6.41	2.05
18	3.56	0.36	0.81	3.26	0.74	1.09	5.86	1.79
22	6.02	0.35	2.41	4.43	1.78	1.57	6.84	1.78
32	7.71	0.39	4.27	5.3	2.94	0.76	6.1	0.60

Age days	Protease-N % dry wt.	Amino-N % dry wt.	Amide-N % dry wt.	Ammonia-N % dry wt.	Nitrate-N % dry wt.	Insol. N/ Water-sol. N
0 (a)	0.47	0.09	0.05	0.005	—	7.71
(b)	0.34	0.10	0.04	0.010	—	8.62
3 (a)	0.27	0.01	0.05	0.02	—	0.38
(b)	0.03	0.09	0.04	0.023	—	0.23
5	0.55	0.33	0.04	0.028	—	1.31
9	0.27	0.64	0.05	0.026	—	1.37
13	0.67	1.25	0.19	0.039	—	2.86
18	1.02	1.54	0.15	0.066	0.27	6.52
22	0.78	0.61	0.13	0.073	0.19	2.01
32	0.55	0.29	0.21	0.027	—	0.67

Over a period of 32 days' growth from seed, it is seen (Fig. 1, Table I) that insoluble N, protein-N and amino-N represent the greater part of the total N. Small quantities of amide-N and ammonia-N occur at all stages but never amount to more than 5 %, and in the earlier stages of growth are only about 1.4 % of the total N.

The most significant fact is the increase of α -amino-acids from 2 % of the total N in the seed to 25 % at the 18th day. This is paralleled by the decrease of insoluble nitrogen compounds, which, after a perplexing maximum at the 3rd day, reach a minimum on the 18th day. Protein, after a rapid fall during the first 3 days of growth, rises to a fairly steady level, representing about 40 % of the total N. The seedling growth of the pea is slower than that of the lupin, and at each stage the plant has a smaller proportion of leaves; hence active synthesis of protein is delayed. Thus the ratio of insoluble to soluble nitrogen compounds falls, and the active metabolites show a tendency to accumulate (Table I). The curve for proteose-N follows closely that for coagulable protein, as would be expected, since this fraction in the pea contains part of the actual protein (legumin). Amides never accumulate. They are present to the extent of 1 % in the seed, and keep at this level through the early stages of growth, showing a tendency to increase in the leafy seedling. Ammonia follows a parallel course but in still less amount. Nitrates have been detected at the 18th and at the 22nd day. Thus the behaviours of the labile metabolic products, other than asparagine and amino-acids, in the lupin and the pea show a close parallel.

Amino-acids represent the chief nitrogenous metabolic products in the pea during the early stages of growth, when hydrolysis of protein is the principal metabolic process. After the 18th day leaf formation accelerates and amino-acids are removed. Comparison with the lupin [McKie, 1931] shows that the rôle of amino-acids in the pea is played by asparagine in the lupin. The amide content of the pea is at all times negligible and asparagine has never been detected. On the other hand, the amino-N content of the lupin never reaches 1 % of the total-N.

Etiolated pea seedlings, grown in complete darkness to the 18th day, show that amino-acids still represent the major water-soluble nitrogen compounds

Table II. *Effect of etiolation on nitrogen distribution.*

	9 days		18 days	
	Etiolated	Green	Etiolated	Green
Ammonia-N (% total N)	0.6	0.48	0.96	1.1
Amide-N (% total N)	3.7	1.05	13.4	3.4
Amino-N (% total N)	5.6	12.01	20.06	25.3
Complex N	—	—	65.6	58.9
Water-sol. N	—	—	34.4	33.4
Complex N	—	—	65.6	58.9
Amide-N	—	—	13.4	3.4
Amino-N	—	—	1	1
Amide-N	—	—	0.67	0.13

(Table II). Thus, while the ratio of complex N to water-soluble N is approximately the same in both types, indicating that light does not appear to affect the extent of hydrolysis, the ratio of amino-N to amide-N changes from 1/0.13 in light to 1/0.67 in the dark, showing that the course of hydrolysis is considerably different. Total N (as % dry weight) is parallel in etiolated and green plants to the 9th day, but is 5 % in the former as compared with 6 % in the latter at the

18th day. If this difference is real—it is difficult to ensure that growth is exactly parallel—it might indicate that nitrogen metabolism, as well as following a different course, is lowered by virtue of decreased intake.

Evidence has been adduced [McKie, 1931], in line with other workers, that asparagine, when it is produced as a nitrogen metabolite, is probably a soluble, mobile translocation product. In the absence of asparagine, amino-acids seem to serve predominantly for translocation.

There is some evidence [Allison, 1934; Maskell and Mason, 1930; Montiverdi, 1889; Prianschnikov, 1922, 2; 1924; Schulze, 1901] that the nature of the translocation products is affected by the concentration of carbohydrate, *i.e.* asparagine formation seems to be greatest in seedlings which have low non-nitrogenous reserve, and this is now substantiated by the comparison of green and etiolated seedlings in the pea. Failing an adequate carbohydrate supply oxidation of amino-acids occurs, with separation of ammonia, which is then transformed into asparagine (*vide* the suggestive finding of Butkewitsch [1909] that, in an asparagine-forming plant, ammonia accumulates if asparagine formation is inhibited by anaesthetising). In the pea, a non-asparagine plant, carbohydrate reserve is comparatively high and amino-acids accumulate until protein synthesis becomes active. The occurrence of asparagine in the lupin is strikingly accompanied by a low carbohydrate reserve. Further, when carbohydrate formation is artificially hindered by absence of light, the amino-acid content falls and is accompanied by amide formation. Thus, in the first days of growth, when the carbohydrate values are identical, no difference is observed between the green and the etiolated seedling. Only when secondary carbohydrate supply, available to the green plant by photosynthesis, fails does oxidation of amino-acid and formation of amide become evident.

SUMMARY.

1. Soluble and insoluble protein-N in the pea together account for about 87 % of the total N of the seed, as compared with 100 % in the lupin. Small quantities of proteoses, amides and amino-acids are also present.
2. Insoluble nitrogen compounds reach a minimum on the 18th day, corresponding with a maximum content of amino-acids.
3. Amino-acids appear to represent the chief metabolic product and to have the same significance in the pea as does asparagine in the lupin.
4. The possible rôle of asparagine as a labile translocation product, formed in plants with low carbohydrate reserve by the oxidation of amino-acids, is confirmed by the comparison of green and etiolated seedlings. Amide is produced at the expense of amino-acids when carbohydrate is depleted by the absence of light.

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CXLVI. A TANNIN FROM THE INDIAN GOOSE-BERRY (*PHYLLANTHUS EMBLICA*) WITH A PROTECTIVE ACTION ON ASCORBIC ACID.

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IN a communication on the ascorbic acid content of Indian plant materials [Damodaran and Srinivasan, 1935], attention was drawn to the fact that different plant juices showed marked variations in the rate of oxidation of the ascorbic acid present in them. As a striking instance mention was made of the fruit of the Indian gooseberry (*Phyllanthus emblica*), which according to the Tillmans-Harris titration method had an ascorbic acid content of about 4-6 mg. per g. of the material. The undiluted press-juice of this fruit was extremely resistant to atmospheric oxidation and, in contrast to most other plant juices which rapidly deteriorated in their ascorbic acid content on standing, retained its titre undiminished even after a week's exposure to the air. The suggestion was therefore made that *Phyllanthus emblica* possibly contained some mechanism which prevented or retarded the oxidation of ascorbic acid. Further evidence for this view was found in the fact that in the gooseberry a considerable proportion of the ascorbic acid remained unaffected even when the fruits were dried. The existence of such a protective mechanism in animal tissues has been demonstrated by Mawson [1935] who, however, could find no evidence of its counterpart in plants.

The distinguishing characteristics of the gooseberry press-juice in comparison with most other plant juices examined were a strong acid reaction and the presence of a large amount of tannin substances with characteristic colour reactions, which were not quantitatively precipitated from solution by normal lead acetate, but which could be completely removed by means of mercuric acetate. Experiments in which the rate of oxidation of the press-juice was compared with that of a solution of ascorbic acid of similar concentration and at the same p_H showed that the stability of the former was not primarily due to its acidity (Table I).

It was also shown that after the complete removal from the gooseberry juice of substances reacting with ferric chloride by precipitation with mercuric acetate the filtrate underwent rapid oxidation approximately at the same rate as a solution of ascorbic acid of the same strength (Table II).

Experiments carried out by making additions of various fractions from the material precipitated by mercuric acetate to solutions of ascorbic acid or to orange juice indicated that the factor responsible for the protective action was either a tannin or associated with the tannins, but clear-cut results could not be obtained on account of the difficulty of preparing homogeneous solutions of the different fractions. It was therefore decided to prepare the tannins in as pure a condition as possible before studying their effects on the oxidation of ascorbic acid.

Serious difficulty was encountered in working with the press-juice on account of its tendency to rapid decomposition with the deposition of a white precipitate which was later identified as ellagic acid. This decomposition could not be prevented either by boiling the liquid to prevent enzyme action, by neutralising the natural acidity of the press-juice or by keeping it in a reducing atmosphere by saturation with sulphur dioxide. The tannins were, however, finally prepared by drying the gooseberry and extracting the powdered material with organic solvents. By fractional precipitation of a methyl alcoholic extract with ethyl acetate and acetone in succession two preparations were obtained representing the chief tannins of the gooseberry. The first of these preparations, soluble in methyl alcohol but not in ethyl acetate, resembled the typical tannins in being precipitated by gelatin and hide-powder, but did not give any of their characteristic colour reactions with ferric chloride. On acid hydrolysis the substance yielded ellagic acid and glucose. The second preparation, which was soluble in ethyl acetate but not in acetone, answered all the tannin reactions and gave rise on hydrolysis to gallic acid in addition to ellagic acid and glucose. As in the case of the majority of the tannins hitherto investigated, it has not been possible to establish the individuality by crystallisation of either of the above preparations or to account quantitatively for the products of their hydrolysis. But care was taken to ensure the complete absence of substances other than of the tannin class, in particular, of sulphur compounds and of tannin degradation products such as free phenols and phenolic acids.

Of the two substances isolated it was found that whilst the simpler one consisting of ellagic acid and glucose had no inhibitory action on the oxidation of ascorbic acid (Table III), the second product composed of glucose, gallic acid and ellagic acid exercised a definite retarding influence on the reaction (Table IV).

Experiments carried out at different hydrogen ion concentrations and in the presence of traces of copper threw some light on the nature of the protective action. It was found that the tannin had no protective action on ascorbic acid at alkaline reaction (Table IV), and that the loss of activity was not due to any appreciable decomposition of the tannin itself at an alkaline reaction. The explanation of this is probably to be found in the fact that the oxidations of ascorbic acid in acid and alkaline media are essentially different reactions [Herbert *et al.*, 1933]. Further, as in the experiments of Kellie and Zilva [1935] on the catalytic action of metals on the oxidation of ascorbic acid, it was found (Table VI) that the protective action could be completely neutralised by the addition of suitable amounts of copper salts, so that it is to be inferred that the action of the tannin under consideration is not a specific effect on the ascorbic acid but is probably connected with its capacity to inhibit the action of heavy metals which are now known to be responsible for the autoxidation of ascorbic acid.

It is also to be stated that all tannins do not necessarily have the type of protective action now noted, as experiments with gallotannin (B.D.H. tannic acid) proved (Table VII). To what peculiarity of structure the tannin from gooseberry owes its action, it is not possible to say at present. It is hoped that experiments now in progress will give a clearer insight into the molecular composition of the gooseberry tannin.

The fruit of *Phyllanthus emblica* has always been held in very high esteem in Indian medicine. As no alkaloidal or other active principle has been demonstrated in it, it seems justifiable to conclude that it owes its medicinal attributes to vitamin C and the protective tannin now described, which make it a valuable antiscorbutic in the dry as well as in the fresh condition. It is interesting to

find it on record that an attack of scurvy in the Indian army at Nassirabad in Rajputana in 1837 was successfully treated with an extract of the dried fruits [Chick and Hume, 1919].

EXPERIMENTAL.

Methods.

Ascorbic acid was determined by the Tillmans-Harris technique [Harris *et al.*, 1933]. The press-juice was obtained from the pulp of the fruit separated from the seeds, metallic contamination being excluded as far as possible during the pounding and pressing.

Oxidation of gooseberry press-juice compared with that of ascorbic acid at the same p_H .

20 ml. of centrifuged gooseberry press-juice filtered through paper-pulp were made up to 100 ml. and titrated against dichlorophenolindophenol. An ascorbic acid solution of nearly the same reducing power was prepared and its reaction adjusted to that of the press-juice (p_H 2) by the addition of very dilute acetic acid. Equal volumes of the two solutions were allowed to stand in large stoppered flasks and the titres of the two against $N/200$ Tillmans's indicator were determined at intervals.

Table I. *Oxidation of ascorbic acid and gooseberry press-juice at the same p_H .*

Time in hours	Ascorbic acid present in 5 ml. (ml. of $N/200$ Tillmans's indicator)	
	Press-juice	Ascorbic acid
0	7.75	6.80
1	7.35	6.35
2	7.10	5.70
4	6.45	4.75
6	5.75	3.55
10	3.90	1.45

Oxidation of press-juice after treatment with mercuric acetate.

25 ml. of centrifuged and filtered press-juice were neutralised with dilute NaOH. A filtered mercuric acetate solution (20 g. in 100 ml. of water) was added till precipitation was complete. The precipitate was separated on the centrifuge, the supernatant liquid freed from mercury by hydrogen sulphide and the filtrate freed completely from hydrogen sulphide by a current of carbon dioxide. The resulting solution, neutralised with dilute NaOH, was made up to

Table II. *Oxidation of ascorbic acid and of gooseberry press-juice after treatment with mercuric acetate.*

Time in hours	Ascorbic acid present in 5 ml. (ml. of $N/200$ Tillmans's indicator)	
	Press-juice	Ascorbic acid
0	6.20	5.95
1	4.95	4.70
2	3.70	3.45
3	2.45	2.25
4	1.25	1.10
5	0.90	0.75
6	0.10	0.00

100 ml. The titres of the solution at different intervals against *N*/200 2:6-dichlorophenolindophenol are compared with those of an ascorbic acid solution at the same neutral reaction in Table II.

Preparation of the tannins.

The pulp of fresh gooseberries was separated from the hard seed by pounding in a stone mortar, dried rapidly at 60° and the dry material reduced to fine powder in a mechanical grinder. 1.5 kg. of the powder were extracted in a Soxhlet extractor with 4 l. of a mixture of equal volumes of dry ether and light petroleum for 6 hours to remove chlorophyll, free gallic acid and other phenolic substances. Extraction was then continued with 4 l. of 98 % methyl alcohol, the air in the apparatus being displaced by carbon dioxide. The methyl alcoholic solution was concentrated under reduced pressure in an atmosphere of carbon dioxide to a syrupy consistency. The light brown concentrate was next shaken up repeatedly with light petroleum to remove the last traces of chlorophyll and with ether to remove any gallic acid present. To the syrup ethyl acetate was gradually added in excess, while the precipitate was removed by centrifuging and purified by dissolving in 98 % methyl alcohol and reprecipitating with ethyl acetate. This process was repeated three times and the product obtained as a white amorphous powder on drying over sulphuric acid *in vacuo*; yield 19 g. (tannin I).

The solution after removal of the first precipitate was concentrated *in vacuo* to nearly 100 ml. Dry acetone was added to this concentrate with stirring. The white precipitate was removed on the centrifuge and purified by dissolving in ethyl acetate and reprecipitating with acetone three times. The substance when dried *in vacuo* over sulphuric acid was obtained as a deliquescent white powder and weighed 13 g. (tannin II).

Experiments with tannin I.

Properties and composition. The fraction precipitated by ethyl acetate was a dry amorphous powder with a faint bitter taste. It dissolved readily in cold water giving a distinctly colloidal solution, which on standing deposited a white crystalline precipitate. The same crystalline material also separated out when some of the substance was hydrolysed at 100° with *N*/20 H_2SO_4 in a pressure-bottle for 6 hours. The insolubility of the material in all solvents other than pyridine and hot alcohol, the appearance of the crystals under the microscope, the characteristic crimson colour on treatment with concentrated nitric acid and dilution showed that the substance in question was ellagic acid. On recrystallisation from pyridine and washing with hot alcohol, yellowish white spindle-shaped crystals, m.p. above 360°, were obtained. The material was further identified by acetylation when it gave an acetyl derivative melting at 339° [Nierenstein, 1909]. The filtrate from the ellagic acid was found to contain a reducing sugar which was identified as glucose by means of the osazone. In a quantitative experiment 0.57 g. of the substance on hydrolysis yielded 0.33 g. of ellagic acid determined by direct weighing and 0.20 g. of glucose as estimated by Bertrand's method. These account for 91 % of the weight of substance taken and are roughly in the proportion of one molecule of ellagic acid to one molecule of glucose. The highly colloidal nature of the solution of the substance however points to a much higher molecular weight. The substance did not show any of the colour reactions with iron salts used for the detection of tannins but was readily precipitated by gelatin and hide-powder. It did not reduce Tillmans's reagent but readily reacted with iodine solution.

Effect on the oxidation of ascorbic acid. A 3% solution of the substance was added to a solution of ascorbic acid and the Tillmans titre value of the mixture compared with that of a solution of ascorbic acid, observations being made every hour (Table III). The results indicated that the substance had no inhibitory action on the oxidation of ascorbic acid.

Table III. *Oxidation of ascorbic acid in the presence of tannin I.*

	ml.	ml.	ml.
Ascorbic acid	20	20	20
Tannin solution	20	10	0
Water	0	10	20
Time in hours	Ascorbic acid present (ml. of <i>N</i> /200 Tillmans's reagent)		
0	4.70	4.95	4.55
1	3.45	3.70	3.35
2	2.20	2.55	2.20
3	1.00	1.20	1.05
4	0.45	0.65	0.35
5	0.05	0.15	0.00

Experiments with tannin II.

Properties and composition. This was a white powder with an astringent taste and extremely deliquescent in air. On heating it did not melt but softened and decomposed at 120° with the liberation of carbon dioxide. It was readily soluble in water, alcohol and ethyl acetate. The aqueous solution on standing deposited insoluble ellagic acid. That the formation of this precipitate was not hindered even by saturating the solution with sulphur dioxide showed that the ellagic acid must have been an integral part of the molecule and did not arise from the oxidation of gallic acid.

With ammonium acetate and ferric citrate a solution of the substance gave a deep violet-coloured precipitate [Warr, 1924]. A dilute solution with a few crystals of potassium nitrite exhibited the gradual change of colour from light pink to deep blue described as characteristic of ellagitannins [Procter, 1894]. The substance was precipitated by gelatin and hide-powder. After hydrolysis of the substance with *N*/20 H_2SO_4 at 100° in a pressure-bottle for 6 hours the decomposition products were identified as gallic acid, ellagic acid and glucose; it thus resembles in composition the tannin studied by Schweitzer [1935]. The ellagic acid separated out as an insoluble precipitate. The filtrate was repeatedly shaken up with ether. The ether on evaporation gave a crystalline residue of gallic acid, which after recrystallisation from water was obtained as fine needles melting with decomposition at 239° and gave the characteristic colour reactions with dilute potassium cyanide and ferric chloride. The aqueous layer after ether extraction was found to contain only glucose identified by the osazone. The tannin has a slight though definite action on Tillmans's reagent, 1 g. of the material being equivalent to 0.0023 g. of ascorbic acid. This reducing action is not affected by repeated solution and reprecipitation of the tannin. Careful tests did not reveal the presence of any sulphur compound.

Effect on oxidation of ascorbic acid. 20 ml. of *N*/100 ascorbic acid were placed in each of three similar flasks of 100 ml. capacity, and 20 ml. of a 3% solution of the tannin added to each. The reactions of the three solutions were adjusted to p_{H} 2, 6 and 10 respectively and the total volume of each made up to 50 ml. For each p_{H} control solutions were prepared from which the tannin was omitted, and the rate of oxidation studied. The results are expressed in mg. of ascorbic acid oxidised to show the effect more clearly (Table IV).

Table IV. *Oxidation of ascorbic acid at various p_H values in the presence of tannin II.*

Time in hours	mg. of ascorbic acid oxidised					
	p_H 2		p_H 6		p_H 10	
	Exp.	Control	Exp.	Control	Exp.	Control
1	0.12	0.28	0.27	0.78	0.81	1.59
2	0.21	0.57	0.48	1.41	1.61	2.40
3	0.36	0.87	0.78	2.22	2.50	2.82
4	0.45	1.11	1.05	2.91	2.97	3.05
5	0.60	1.32	1.35	3.27	2.97	3.45
6	0.77	1.56	1.58	3.54	2.97	3.45

Oxidation of the tannin in alkaline solution. It was thought necessary to examine whether the loss of protective action at p_H 10 was due to oxidation of the tannin. A solution of the tannin of the same concentration as used in the previous experiments was prepared. Equal volumes of it were taken in three similar flasks and their p_H values adjusted to 2, 6 and 10 respectively. The iodine titres of these solutions which serve as an index of their tannin contents were determined each hour. 1 ml. of the solution was withdrawn each time, treated with 5 ml. of $N/200$ iodine solution and after mixing well the excess of iodine titrated back against $N/200$ thiosulphate solution. The results in Table V show that the oxidation of the tannin at p_H 10 is too slight adequately to account for its loss of protective action.

Table V. *Oxidation of tannin II at different p_H values.*

Time in hours	ml. of $N/200$ iodine solution		
	p_H 2	p_H 6	p_H 10
0	2.85	3.05	2.90
1	2.80	2.95	2.75
2	2.85	3.00	2.50
3	2.85	2.90	2.45
4	2.75	2.80	2.30
5	2.70	2.90	2.20
6	2.75	2.85	2.05

Effect of adding copper sulphate on the protective action. An approximately 0.03 % solution of ascorbic acid and exactly 3 and 0.05 % solutions of the tannins and pure copper sulphate respectively were prepared in water obtained free from metallic contamination by repeated distillation over glass [Kellie and Zilva, 1935]. Six conical flasks of 50 ml. capacity were taken and numbered.

Table VI. *Oxidation of ascorbic acid in the presence of tannin II and copper sulphate solution.*

	ml.	ml.	ml.	ml.	ml.	ml.
Ascorbic acid (0.03 %)	20	20	20	20	20	20
Tannin solution (3 %)	20	20	20	20	20	0
Copper sulphate (0.05 %)	1	2	3	4	5	0
Water	4	3	2	1	0	25
Time in hours	Ascorbic acid present (ml. $N/200$ Tillmans's reagent)					
0	4.70	4.85	5.10	4.80	5.05	4.95
1	4.65	4.70	4.80	4.30	4.40	4.75
2	4.60	4.65	4.70	4.20	4.35	4.65
3	4.55	4.60	4.60	4.10	4.15	4.50
4	4.35	4.30	4.45	3.90	3.85	4.30

The solutions were added to each in the proportions given in Table VI. 2 ml. of the solution in each case were titrated against *N*/200 Tillmans's reagent every hour. The last column is the control with neither tannin nor copper sulphate added.

Commercial gallotannin exercises no protection (see Table VII).

Table VII. *Oxidation of ascorbic acid in the presence of gallotannin at p_H 6.*

Time in hours	Ascorbic acid present (ml. of <i>N</i> /200 Tillmans's reagent)		
	20 ml. ascorbic acid + 20 ml. 3% tannin	20 ml. ascorbic acid + 20 ml. 1.5% tannin	20 ml. ascorbic acid + 20 ml. water
0	6.25	5.80	5.35
1	6.10	5.35	5.05
2	5.85	4.80	4.75
3	5.60	4.30	4.25
4	5.15	4.05	4.00
5	4.70	3.85	3.75
6	4.30	3.55	3.60

SUMMARY.

1. The atmospheric oxidation of ascorbic acid in the press-juice of the fruits of the Indian gooseberry (*Phyllanthus emblica*) is considerably slower than that of a solution of pure ascorbic acid at the same p_H .

2. Two different tannins have been prepared from the fruits and their properties studied.

3. One of these containing gallic acid, ellagic acid and glucose in its molecule is shown to have an inhibitory action on the oxidation of ascorbic acid solutions in air and is apparently responsible for the resistance to oxidation of the ascorbic acid in gooseberry press-juice.

4. The other tannin isolated, consisting of ellagic acid and glucose, exercises no such protective action and resembles in this respect commercial gallotannin.

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CXLVII. STUDIES ON BILE PIGMENTS.

III. THE QUANTITATIVE DETERMINATION OF UROBILIN AND UROBILINOGEN IN URINE AND FAECES.

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In a previous paper [Naumann, 1936] the qualitative tests for urobilinogen and urobilin have been studied. In the following the quantitative aspect of the problem is treated.

A basis for the estimation of urobilin and related compounds was first introduced by Terwen [1924; 1925]. The main features of this method consist in determining the sum of urobilinogen and urobilin as urobilinogen after reduction by means of a ferrous salt in alkaline solution, the extraction of the total chromogen with ether or light petroleum, the timing of the aldehyde reaction, the replacement of the hydrochloric acid by acetic acid by adding sodium acetate, and the comparison with a phenolphthalein standard in an Autenrieth colorimeter. The method has been examined and partly modified by Greppi [1926], Fuerth and Singer [1929], Heilmeyer [1931], Watson [1931], Royer [1934] and Christophe [1934].

Terwen's method is subject to the disadvantage of the non-specificity of the aldehyde reaction as explained formerly. Many of the interfering substances, *e.g.* indole and phenol derivatives, phenazone, Extr. Rhei *etc.*, are extracted by ether and light petroleum; the replacement of the hydrochloric acid by acetic acid only partly eliminates this source of error. A further disturbance is caused by the presence of much bilirubin which is extracted partly by ether, whilst light petroleum does not extract bilirubin, but at the same time fails to extract urobilinogen completely. Also the final colour in the presence of interfering substances is more orange or brownish, which renders the colorimetric comparison rather difficult.

EXPERIMENTAL.

Influence of protein and mucus on the ether extraction.

Yet a further source of error caused by the presence of protein in urine is the incompleteness of the urobilinogen extraction (see Table I). If the albuminuria is not excessive the loss can be presumed to be less than 20%. A much greater

Table I. *Influence of the presence of protein and mucus on the extractability of urobilinogen.*

Amount of urine tested	Approx. % of protein	Urobilinogen content in mg./100 ml.	Loss %
10 ml. urine without addition	—	0.083	—
10 ml. urine + 0.1 ml. serum	0.1	0.072	13
10 ml. urine + 0.5 ml. serum	0.5	0.068	18
10 ml. urine + 1.0 ml. serum	1	0.065	22
10 ml. urine + 2.0 ml. serum	2	0.062	25
10 ml. urine + 1.0 ml. sputum	—	0.054	35

loss is caused by the presence of mucus which in a rather small quantity can cause a deficiency of 35 %. Thus the presence of protein disturbs in two ways: (1) by interfering with the aldehyde reaction; (2) by preventing complete extraction of the urobilinogen.

The reduction of urobilin by ferrous ammonium sulphate (Mohr's salt).

Greppi [1926] states that the reduction is not always successful but will be effective after repetition, and Adler [1927] and Royer [1934] point out that urobilinogen is partly destroyed during reduction, whilst the latter found that if ferrous sulphate is used according to Watson [1931] there is no destruction. If the iron reduction is to be effective the following conditions must be ensured:

- (1) Bilirubin is not transformed into urobilinogen.
- (2) Urobilinogen is not destroyed.
- (3) Urobilin is reduced completely.

Watson [1931] states that bilirubin is partly reduced to urobilinogen when ferrous sulphate is used and removes the bilirubin with $\text{Ca}(\text{OH})_2$ before reducing. However, under the conditions of Terwen's method and the modification described below no positive aldehyde test was obtained either directly or after extraction with light petroleum, although the bilirubin solutions showed a lighter colour after reduction. It can thus be assumed that with Mohr's salt reduction of bilirubin to urobilinogen does not take place.

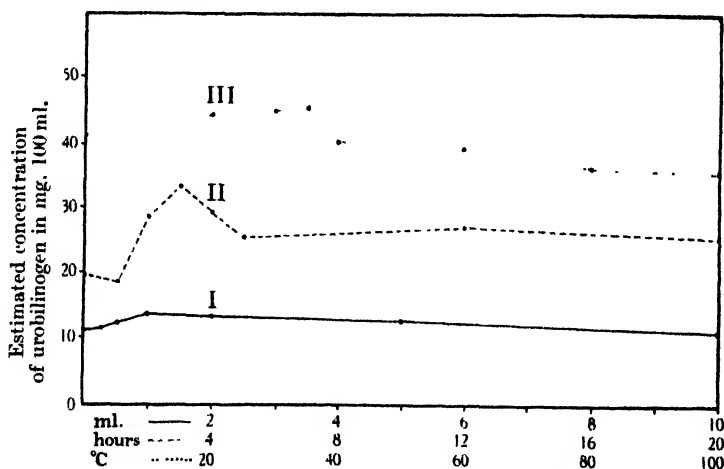


Fig. 1. Reduction of aqueous faecal extract with Mohr's salt under varying conditions of concentration, time and temperature.

In order to investigate whether urobilinogen is destroyed and whether urobilin is completely reduced it was necessary to determine the conditions of reduction. Fig. 1 shows the effect on reducing experiments with aqueous faecal extracts of variation of the concentration of Mohr's salt solution (Curve I), of the time of reduction (Curve II) and of the temperature (Curve III). In the curves I, II and III the estimated amount of urobilinogen is plotted against ml. of Mohr's salt solution added to 2 ml. of faecal extract, against time of reduction, and temperature in $^{\circ}\text{C}$. of the reaction mixture respectively. From these curves the following optimum conditions are deduced:

- (1) The optimum concentration is 2 volumes of diluted faeces + 1 volume of Mohr's salt solution.

(2) The optimum time of reduction is 3 hours at 20°.

(3) The optimum temperature is 35° for 5 min. of reduction.

However, as will be seen below these results are not always uniform.

Table II. *Comparison of different reducing methods.*

		Without reduction	Terwen's method	Watson's modification	Author's modification	
					3 hours	5 min. 35°
Urine No.	1	0.12	0.10	0.07	0.14	0.11
	2	0.33	0.26	0.21	0.33	0.32
	3	0.37	0.35	0.22	0.45	0.17
	4	0.75	0.81	0.84	0.92	0.73
	5	2.17	1.90	2.22	2.86	2.01
Faeces No.	1	11.65	11.88	13.38	15.41	18.00
	2	28.60	33.80	36.40	34.40	35.30
	3	33.10	40.80	35.40	38.30	34.40
	4	106.50	83.40	96.00	96.30	108.20
	5	333.50	247.30	396.20	284.50	388.30

Technique of tests.

From these results the following technique has been derived.

Test in faeces.

1 g. of the well mixed faeces is weighed on a microscope slide coverslip and ground together with the glass in a mortar with gradual addition of 9 ml. of water. 3 tests are then performed.

(a) *Test without reduction.* 0.2 ml. of the faecal mixture is washed quantitatively into 5 ml. of water placed in a separating funnel; 20 ml. of ether and 0.5 ml. of 20% tartaric acid are added. After shaking vigorously the aqueous phase is drawn off and the ether washed twice with small amounts of water. Then 5 drops of 1% *p*-dimethylaminobenzaldehyde in conc. HCl are added and shaken for 1½ min. 1 ml. of conc. sodium acetate is added and the coloured solution drawn off into a 10 ml. measuring cylinder, the ether washed with 20% acetic acid and the solution made up to 5 or 10 ml. or a greater volume according to the colour depth and compared with Terwen's phenolphthalein standard in an Autenrieth colorimeter.

To the remaining faecal mixture, 2.5 ml. of 16% ferrous ammonium sulphate and 2.5 ml. of 12% NaOH are added, well mixed and poured into two small test-tubes.

(b) *Test after reducing for 3 hours.* One of the two portions covered with a layer of paraffin oil is set aside in the dark for 3 hours and filtered. 0.2 ml. of the filtrate is worked up as described above.

(c) *Test after reducing for 5 min.* One of the two portions is put for 5 min. in a water-bath at 35° and then filtered immediately. 0.2 ml. of the filtrate is treated as described.

Calculation.

(a) $100 - x \times \frac{\text{final volume}}{0.2} \times 10 \times 0.4$ is the concentration of urobilinogen in mg./100 g. in the faeces without reduction, i.e. the actual urobilinogen content without urobilin. x is the reading of the scale of the Autenrieth colorimeter.

(b) and (c). $100 - x \times \frac{\text{final volume}}{0.2} \times 1.5 \times 10 \times 0.4 + 2\%$ (to allow for the 0.2 ml. taken away for (a) from the original faecal mixture) is the concentration of urobilinogen in mg./100 g. in the faeces after reduction, i.e. the concentration of urobilinogen + urobilin.

Test in urine.

(a) *Test without reduction.* 2 ml. of urine are placed in a separating funnel and treated correspondingly as described above.

(b) *Test after reduction.* To 9 ml. of urine are added 0.5 ml. of 16% ferrous ammonium sulphate and 0.5 ml. of 12% NaOH with good mixing. The mixture, placed in a small test-tube and covered with a layer of paraffin oil, is set aside for 3 hours in the dark and filtered. 2 ml. of the filtrate are treated as described.

Calculation.

(a) $100 - x \times \frac{\text{final volume}}{2} \times 0.4$ is the concentration of the actual urobilinogen in mg./100 ml. in the urine, if x is the reading on the Autenrieth colorimeter.

(b) $100 - x \times \frac{\text{final volume}}{2} \times 1.11 \times 0.4$ is the concentration of urobilinogen + urobilin in mg./100 ml. in the urine.

A comparison of the different reduction methods in Table II shows a 10–50 % better yield by the author's modification with the exception of faeces samples Nos. 2, 3 and 5. It must be considered, however, that differences up to 5 % are within the limit of error. The advantage of the described technique is more pronounced in the urine tests where only 3 hours' reduction gives good results.

From Table II it is seen that the figures obtained by Terwen's method in all the urine samples except No. 4, and in two of the faeces samples are lower than those obtained without reduction, whilst the figures obtained with Watson's modification are lower only in three urines and one faeces sample. This can be explained only by a destructive action of the reducing process on the urobilinogen. On the other hand the 3 hours' reduction described gives a higher figure than is obtained without reduction in all urine samples except No. 2, where the figures are the same, and in the faeces samples figures obtained with either the 3 hours or the 5 min. technique are higher than without reduction. In the latter case the better yield must be considered to be the correct result. It must therefore be concluded that Terwen's method and to a smaller extent also Watson's modification effect a partial destruction of urobilinogen, which is prevented or at least greatly restricted by the technique described here.

The completeness of reduction of the urobilin is usually estimated by the spectroscopic test which depends greatly on the sensitivity of the instrument. A distinct urobilin band in Hartridge's spectroscope after reduction by either of the described methods has not been detected and has only been observed in a malaria case with an excessive excretion of urobilin. The problem has been investigated further by the following experiment.

A purified aqueous urobilinogen solution was prepared according to Terwen by treating a urobilinogen light petroleum extract with slightly alkaline water. The urobilinogen content of the almost colourless solution was 3.83 mg./100 ml. After standing for 24 hours in the dark the solution became slightly brown with a distinct urobilin spectrum and a urobilinogen content of 2.61 mg./100 ml. After reduction according to the author's modification the filtrate was only faintly yellow without a urobilin spectrum. The content was 3.84 mg./100 ml.

The latter experiment which is in accordance with similar experiments of Terwen supports the assumption that in general the reduction of urobilin will be complete unless a great excess of the pigment is present. In such cases faeces or urine must be diluted or a greater amount of iron solution used.

Stability of the phenolphthalein standard solution.

A disadvantage of Terwen's original method is the constant necessity of fresh preparation of the standard phenolphthalein solution which is supposed to keep stable not longer than a few hours. Observation of the phenolphthalein standard, prepared by dilution of 1 ml. of 0.05 % alcoholic phenolphthalein and 5 ml. conc. Na_2CO_3 plus distilled water up to 100 ml., showed however that the decrease of colour of such a solution takes place in a certain regular way. After a period of stability of about 5 hours the colour strength decreases slowly until a new stability is reached after about 18 hours. This definite value is maintained for about a month and decreases only very little during the course of a year.

This change is due to the influence of OH ions on the different compounds of the phenolphthalein group and is a general property of all phthaleins as pointed out by Kolthoff and Fischgold [1932]. The degree of decrease is about 15–30 % of the colour of the freshly prepared solution and depends on the purity of the phenolphthalein preparation. In view of the regular nature of this change it is possible for practical colorimetric purposes to use the phenolphthalein standard as a relatively stable one after an interval of a day provided that it is controlled at intervals of about a month. Such a phenolphthalein solution has been kept by the author in the wedge of the Autenrieth colorimeter for 17 months and no change has been noticed for about 8 months.

SUMMARY.

1. The quantitative estimation of urobilinogen and urobilin by the method of Terwen has been examined. It has been found that errors are caused by the unspecificity of the aldehyde reaction owing to interfering substances, particularly at a low urobilinogen content, and by the fact that the extraction is not complete in the presence of protein and mucus.

2. Investigation of Terwen's method of reducing urobilin by means of Mohr's salt has given the following results:

(a) The reduction is dependent on the time factor, the reducing action following a course with a peak after 3 hours at 20° or 5 min. at 35°.

(b) The reduction is dependent on a suitable relation between iron salt and urobilin concentration.

(c) In the course of the reduction urobilinogen is partly destroyed.

(d) The reduction is complete except in the presence of a great excess of urobilin.

(e) Bilirubin is not reduced to urobilinogen by Mohr's salt.

3. A modification of the reduction by using a suitable concentration of Mohr's salt and limiting the time of the reducing process gave 10–40 % better yields than the method of Terwen and Watson.

4. The phenolphthalein standard solution fades to the extent of 15–30 % during 18 hours after preparation and can be regarded afterwards as relatively stable for the period of a year provided that it is controlled at monthly intervals.

I wish to record my best thanks to Dr Arthur Davies, Director of the Devonport Pathological Laboratories of Seamen's Hospital, Greenwich, for his help and interest in my work, to the Seamen's Hospital Society for their assistance and hospitality to me and my assistant, Miss Larissa Frenkel, whose co-operation has been of value, and to the Academic Assistance Council for a personal grant.

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CXLVIII. STRUCTURE IN RELATION TO CHROMIC OXIDATION OF NITROGENOUS SUBSTANCES.

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It was noted in an earlier communication [Acharya, 1936] that when nitrogenous substances were treated with a mixture of chromic and sulphuric acids, a part of the organic nitrogen was oxidised to nitrate while another was, in several cases, lost in gaseous form. A more detailed examination showed that the percentage of total nitrogen recovered as ammonia showed a higher and variable value with small amounts of substances but soon reached a constant figure on increasing the weight of substance taken. For amounts of substances of less than 5 mg. nitrogen content there seemed to be an interference, possibly from the high proportion of sulphuric to chromic acid in the oxidising mixture, which tended to raise the percentage recovery to nearly 100; whereas on increasing the weight of substance taken and consequently narrowing the chromic:sulphuric ratio, the curves representing the percentage of total nitrogen recovered as ammonia rapidly became horizontal, and continued to

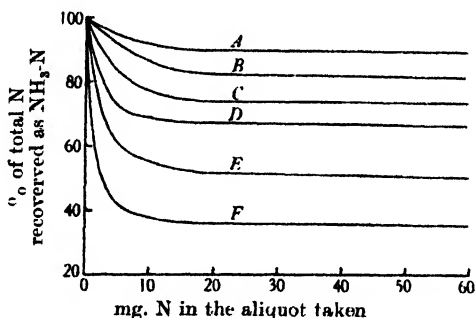


Fig. 1. The relation between oxidation constant and weight of substance taken. *A*, caseinogen; *B*, edestin; *C*, caffeine, theobromine; *D*, urea, creatine, uric acid; *E*, dicyandiamide; *F*, guanidine.

remain at that level. The percentage value at this constant level has been termed the "oxidation constant" [Acharya, 1935] and bears a definite relationship to the structure of the compound. The curves for some typical substances are given in Fig. 1 and are comparable to those obtained by Shewan [1935].

I. Influence of structure on the nitrogenous products obtained.

In order to explore the relationship between structure and the nature and quantity of nitrogenous products obtained, a large number (nearly 150) of organic nitrogenous substances of different types were treated with chromic-sulphuric mixture according to the previous procedure [Acharya, 1936]. In some cases commercial samples of the substances contained chloride either as impurity or

as hydrochlorides of nitrogenous bases, and it has been reported already that chlorides tend to increase the proportion of nitrate-nitrogen at the cost of ammoniacal nitrogen and thus interfere in the determination of the oxidation constants. In such cases the chlorides were removed by precipitation with silver sulphate.

With several substances, it was found that the sum of ammoniacal and nitrate-nitrogen formed accounted only for a fraction of the total nitrogen, the remaining portion being presumably lost in gaseous form. As the apparatus and procedure outlined before [1936] limited itself to the estimation of ammoniacal and nitrate-nitrogen only and no provision was made for the collection and estimation of gaseous nitrogen compounds evolved, the following modified apparatus shown in Fig. 2 was adopted to meet the difficulty.

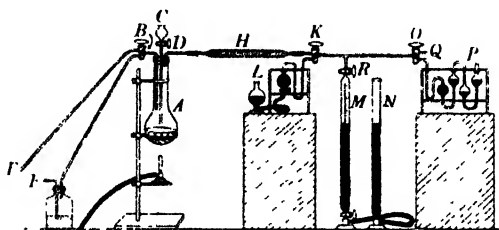


Fig. 2. Apparatus for the estimation of nitrogen by wet combustion.

A is a Kjeldahl flask of about 300 ml. capacity fitted with an inlet tube *B*, thistle funnel *C* and exit tube *D*. Tube *B* carries a three-way cock connected to an oxygen cylinder at *E* and water suction at *F*. The exit tube *D* is connected to a tube *H* about 20 cm. long and 0.5 cm. bore packed with Sofnolite (to absorb the CO_2 evolved) and thence to a Hempel's burette *M* via the three-way cock *K*. *L* is an explosion pipette, *O* a three-way cock connected to an absorption pipette *P* filled with alkaline pyrogallol, and also to an opening *Q* which serves either as an exit to the atmosphere or as inlet for the admission of hydrogen for purposes of explosion. The burette *M* is filled with mercury and the pressure inside can be manipulated by altering the position of the levelling tube *N*. The connections in the gas analysis apparatus are all of thick walled capillary tubing.

To start with, a known weight of the substance under examination is transferred into the flask *A* through a glass tube, the sides of which are washed with about 5 ml. of water. The flask is fitted into place, the mercury level in *M* is brought to *R* by raising the levelling tube *N*, cocks *R* and *O* are closed and the air inside the apparatus is replaced by oxygen, by alternately applying suction at *F* for a few minutes followed by connecting the tube *B* to the oxygen cylinder *E*. After repeating this operation three or four times, suction is applied at *F* for some minutes to ensure low pressure in the apparatus, the levelling tube *N* is lowered so as to keep the pressure at *R* about 3–4 in. of mercury and cock *R* is opened. The level of mercury in *M* shows a fall on opening *R* but slowly rises on continuing the suction at *F*. When the level reaches *R*, further suction is cut off by closing cock *B*; and chromic acid followed by sulphuric acid is added through the thistle funnel *C*, as described previously [Acharya, 1936]. The sulphuric acid is added in small amounts at a time so as to avoid excessive vigour of reaction and escape of gaseous products by back-bubbling through the thistle funnel *C*. After the addition of acid is completed the contents of the flask are mixed well and heated to gentle boiling with a micro-burner.

On the addition of sulphuric acid there is vigorous decomposition and gas evolution, as shown by the rapid fall of mercury level in *M*. The internal pressure in the apparatus is kept at about 10 in. of mercury by lowering the levelling tube *N* from time to time so as to keep the difference in levels between *M* and *N* at about 20 in. After about 25–30 min. heating (and longer in certain cases, cf. p. 1029), cock *R* is closed, cock *C* is opened, the levelling tube *N* is raised to equalise the levels in *M* and *N* and the volume of the gas collected in *M* is read off. This represents a mixture of oxygen (formed by decomposition of chromic acid) and gaseous nitrogen compounds evolved by the action of chromic acid on the nitrogenous substance taken. These latter could possibly be nitrogen and nitrous oxide only, since nitric acid in the amounts formed is retained in the digest itself by sulphuric acid and any trace of the higher oxides of nitrogen which might be evolved would be retained by the soda-lime tube *H* which, incidentally, also serves to remove the CO_2 formed.

The relative proportions of oxygen, nitrous oxide and nitrogen in the mixture in *M* were determined by two methods.

(1) Oxygen was removed from an aliquot by absorption in alkaline pyrogallol. This removed in the earlier stages some N_2O also, but after a few absorptions further absorption of N_2O was found to be negligible. The residual gas containing nitrous oxide and nitrogen was measured and analysed by explosion with hydrogen. The contraction in volume on explosion was equal to the amount of nitrous oxide present.

(2) An aliquot of the gaseous mixture containing O_2 , N_2O and N_2 was exploded with a known volume of hydrogen, the contraction in volume noted and the amount of residual hydrogen determined by a second explosion with oxygen. If the volumes at N.T.P. of gases present in the given aliquot be $\text{O}_2 = X$, $\text{N}_2\text{O} = Y$ and $\text{N}_2 = Z$, the values of *X*, *Y*, *Z* could be determined from the following equations:

$$X + Y + Z = a = \text{original volume of aliquot};$$

$$3X + Y = b = \text{contraction on explosion with hydrogen};$$

$$2X + Y = c = \text{volume of hydrogen used up in the first explosion}.$$

It was found that the two methods gave concordant results, the first being adopted for simplicity of procedure and consistency of values.

After completion of the gas analysis, the digested residue in the Kjeldahl flask *A* was analysed for ammoniacal and nitrate-N as previously [Acharya, 1936].

The values for nitrogen distribution (*i.e.* $\text{NH}_3\text{-N}$: $\text{NO}_3\text{-N}$: $\text{N}_2\text{O-N}$ and N_2) by chromic oxidation of some typical groups of organic substances are given in Table I.

They show that nitrogenous substances in their relation to oxidation by chromic acid could be broadly subdivided under the following heads:

(1) Substances having their nitrogen atoms attached to different carbon atoms; these yield almost the theoretical recovery of nitrogen in the form of $\text{NH}_3\text{-N}$ plus a small quantity of $\text{NO}_3\text{-N}$ with the exception of hydroxylamine and hydrazine derivatives.

(2) Substances having two or more nitrogen atoms attached to the same carbon atom; these yield low recoveries of nitrogen as $\text{NH}_3\text{-N}$ the proportion depending on the structure of the compound. The proportions of ammonia recovered from certain typical groupings are as follows: biuret group, 2/3 of total nitrogen; iminazole group, 4/5; guanidine group, 4/11; creatine group, 2/3. Of the fraction not recovered as ammonia, a small portion is oxidised to nitrate but the main bulk is lost in gaseous form as nitrous oxide.

(3) Hydroxylamine derivations are almost wholly converted into nitrous oxide and nitrate, while hydrazine derivatives lose almost the whole of their nitrogen as elementary nitrogen. In both cases the proportion of ammonia formed is inappreciable.

Table I.

Types of substances	% of total nitrogen as		
	NH ₃ -N	NO ₃ -N	N ₂ O-N
I. Compounds with N atoms attached to different C atoms:			
(a) Tertiary amines including pyridine, quinoline, isoquinoline and alkaloids; quaternary ammonium derivatives including betaine and choline	100	—	—
(b) Secondary amines and substances with >NH groups	95	5	—
(c) Primary amines, amino-acids, amides and compounds easily hydrolysed to above, <i>e.g.</i> nitriles	97.5	2.5	—
II. Compounds with two N atoms linked to a C atom:			
(a) Compounds with —NH—CO—NH— grouping or easily hydrolysed to the above grouping by acids, <i>e.g.</i> cyanamide, urea, biuret, alloxan, allantoin, uric acid <i>etc.</i>	66.7 (2/3 of total N)	3.3	30
(b) Compounds with $\text{CH} \begin{smallmatrix} \nearrow \text{N} \\ \searrow \text{N} \end{smallmatrix}$ grouping, <i>e.g.</i> glyoxaline, histamine, histidine <i>etc.</i> (values for the α -amino groups have been deducted)	80 (4/5 of total N)	—	20
(c) Substances with both the above groupings in the molecule, <i>e.g.</i> caffeine, theobromine <i>etc.</i>	74	—	26
III. Compounds with three N atoms linked to the same C atom:			
(a) Strongly basic substances absorbing CO ₂ from the air, <i>e.g.</i> guanidine	36.5 (4/11 of total N)	2.5	61
(b) Arginine, deducting α -amino-group	50	2.5	47.5
(c) Creatine, creatinine, dicyandiamide (deducting end —CH group)	66.7 (2/3 of total N)	—	33.3
IV. Other nitrogenous compounds:			
(a) Hydroxylamine and its derivatives	0–5	35–40	As N ₂ O 55–60
(b) Hydrazine and its derivatives	0–5	—	As nitrogen 95–100

II. Resistance of nitrogenous substances to chromic oxidation.

While most nitrogenous substances, *e.g.* aniline, acetamide, hippuric acid, asparagine *etc.*, were rapidly oxidised by the chromic-sulphuric mixture and, under the experimental conditions adopted, theoretical values for carbon were obtained after 20–25 min. digestion, in the case of others, higher and varying degrees of resistance to oxidation were met with. Compounds having two or three nitrogen atoms attached to the same carbon atom, *e.g.* urea, biuret, caffeine, creatine, guanidine, dicyandiamide *etc.*, were more slowly attacked and correct values for carbon were obtained only after about 40–45 min. heating. Pyridine, quinoline and isoquinoline required more than an hour. Betaine, choline and the tetrammonium bases were very slowly attacked. The relative rates of oxidation of different types of nitrogenous substances are shown graphically in Fig. 3 and their possible physiological significance will be discussed later.

Shewan [1935] also noted that a much longer time of heating was necessary to obtain theoretical yields of nitrogen from betaine and quinoline derivatives,

but attributed it to a preliminary transformation of the nitrogen into some form before conversion into ammonia. But a simultaneous comparison of the yields of CO_2 obtained would show that the longer time necessary in these cases is due to resistance to oxidation.

It has been noted already [1936] that completion of oxidation of such resistant compounds could be tested either by weighing the Sofnolite tubes at half-hour intervals or better by having two sets of Sofnolite tubes in parallel and passing the CO_2 into each set alternately while weighing the other.

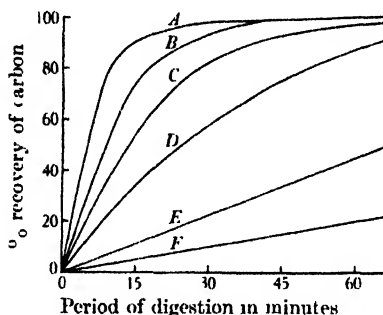


Fig. 3. Rate of oxidation of different nitrogenous compounds. A, aniline; B, pyrrole, piperidine; C, quinoline, *iso*quinoline; D, pyridine; E, betaine, choline; F, $(\text{CH}_3)_4\text{N}.\text{OH}$.

DISCUSSION.

The relation between structure and chromic oxidation of nitrogenous substances has been hinted at by previous workers [Guyot and Simon, 1920; Shewan, 1935], but has not been systematically examined before. The data given above would show that the loss occurs in the form of nitrous oxide in the case of C-2N and C-3N linkages and with hydroxylamine derivatives; whereas free nitrogen is liberated from hydrazine and azo-derivatives. The fraction of total nitrogen recovered as $\text{NH}_3\text{-N}$, or the value of the chromic "oxidation constant", depends on the particular type of linkage, even among the C-2N and C-3N linkages, as shown in Table I.

In regard to the applicability of the chromic acid method for the estimation of nitrogen, the procedure as outlined previously [Acharya, 1936] was found to give only approximate results and the limits of applicability of the method for the determination of nitrogen in soils and plant materials were defined. For organic compounds containing the C-2N and C-3N linkages, or the hydroxylamine and hydrazine groups the method was inapplicable without further modification. The modified procedure given in this paper promises to be one of universal applicability for all types of nitrogenous substances. This improved wet combustion procedure could with advantage replace the traditional Dumas method for nitrogen, in view of its rapidity and ease of manipulation, and could be easily modified so as to make a simultaneous determination of carbon and nitrogen on the same sample.

It is interesting to note that substances which have the C-2N and C-3N linkages exert a marked physiological action on the human and animal systems, *e.g.* purine and pyrimidine bases, guanidine, creatine, dicyandiamide, arginine, histamine, histidine *etc.*, and all of them evolve substantial amounts of nitrous oxide on oxidation. It is not intended, at the present stage, to draw any causal relationship between such physiological activity and the formation of nitrous

oxide—at least until direct experimental evidence is forthcoming on the production of nitrous oxide by biological or enzymic oxidation of the above types of substances. At any rate, the technique of chromic oxidation appears capable of being developed into a rapid laboratory method for the detection of substances exerting marked physiological action.

It is also noteworthy that substances exhibiting a marked resistance to oxidation such as pyridine, quinoline, *isoquinoline* and the tetrammonium bases, *e.g.* betaine and choline, are either of no nutritional value or are highly harmful to the human and animal system. It is well known that administration of choline, betaine or the tetrammonium bases leads to a rapid depletion of the fat content of the liver. A possible explanation for this may be found in the above noted high resistance of these substances to oxidation and the effort made by the system to effect their oxidation and removal even at the cost of a large amount of fat.

The chromic oxidation method promises to be of value in the characterisation of different classes of proteins and in assessing the nutritional value of different types of food materials. It is well known that the quality of a food material improves with its content of diamino-acids, *e.g.* arginine and histidine, and these amino-acids yield a low recovery of $\text{NH}_3\text{-N}$ on chromic oxidation. The values for "oxidation constants" for some typical proteins are given in Table II. There appears to be a rough inverse proportionality between the content of arginine and histidine and the loss of nitrogen on chromic oxidation. A more detailed examination of the behaviour of proteins, more widely differing in their contents of diamino-acids, and of protein hydrolysates and decomposition products towards chromic oxidation may yield information of great interest and value.

Table II.

Substance	Arginine and histidine as % of total nitrogen	Chromic "oxidation constant"
1. Wheat gliadin	9.0	97
2. Wheat gluten	12.2	95
3. Caseinogen	13.6	92.5
4. Glutenin (wheat)	15.0	92.5
5. Egg albumin	16.7	90
6. Blood albumin	17.7	90
7. Haemoglobin	18.8	90
8. Fibrin (blood)	18.7	90
9. Keratin	19.3	89
10. Edestin	32.7	82.5
11. Histidine	100	86.7
12. Arginine	100	66.7

The figures for the content of arginine and histidine are taken from the analytical data presented by Mitchell and Hamilton [1929].

SUMMARY.

1. The qualitative and quantitative composition of the products obtained by chromic oxidation of nitrogenous substances vary with the structure of the compound. Compounds in which the nitrogen atoms are attached to different carbon atoms, with the exception of hydroxylamine and hydrazine derivatives, yield full recovery of nitrogen in the form of $\text{NH}_3\text{-N}$ accompanied by small quantities of $\text{NO}_2\text{-N}$; those having two or three nitrogen atoms attached to the same carbon atom lose a portion of the total nitrogen in the form of nitrous oxide. Hydroxylamine derivatives are converted into nitrous oxide and nitrate,

while hydrazine derivatives yield mainly elementary nitrogen; in both cases, the amount of $\text{NH}_3\text{-N}$ formed is inappreciable.

2. The fraction of total nitrogen recovered as $\text{NH}_3\text{-N}$ is termed the chromic "oxidation constant" and is shown to depend on the structure of the compound.

3. The varying resistance to chromic oxidation of different types of nitrogenous compounds has been studied. Pyridine, quinoline, *iso*quinoline, betaine, choline and the tetrammonium bases are found to be highly resistant; the physiological significance of such resistance has been discussed.

4. An improved wet combustion apparatus has been described which includes the analysis of gaseous products and is applicable to the determination of nitrogen in all types of organic compounds.

5. The potentialities of the chromic acid method in offering a laboratory test for the detection of substances possessing marked physiological activity, in the characterisation of proteins and in assessing the nutritive value of different kinds of food materials have been pointed out.

The author's thanks are due to Sir E. J. Russell and Mr E. H. Richards for their interest in the work.

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CXLIX. THE EFFECT OF VARIOUS FATS IN THE PRODUCTION OF DIETARY FATTY LIVERS.

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THE work described in this paper is the result of a study of the degree to which certain fats of widely varying chemical composition and physical properties accumulate in the liver when fed to animals under certain dietary conditions. It was undertaken in order to obtain information concerning the nature of the fatty acids which appeared in the liver under these different conditions and because knowledge of this kind should provide evidence as to the part played by the chemical nature of the food fat in fatty liver production. Further, it seemed possible that additional evidence of the part played by the liver in fat metabolism might be so obtained. Such a study has not so far been reported, for in investigations of the fatty liver problem, Best and his colleagues have used beef fat and "Crisco" as their dietary fats for the routine production of fatty livers, whilst for the same purpose the former fat only has been used in this laboratory. In the first experiments carried out in 1933 we employed the mixed grain diet with 40% fat which has been much used by the Toronto workers since their original discovery [Best *et al.*, 1932]. Groups of animals were fed for three weeks on this diet with butter, beef dripping, palm oil, coconut oil, olive oil and cod-liver oil as the dietary fats. The average percentages of fat (fatty acids and unsaponifiable matter) present in the livers were 3.25, 5.70, 6.99, 6.33, 6.02 and 3.42 respectively. This and other failures to produce fatty livers by the use of the grain diet with 40% fat caused us to leave the problem at that time and led to the investigations which resulted in the finding that the amount of fat occurring in the liver was governed by the amount of protein in the diet, irrespective of any action of choline [Channon and Wilkinson, 1935; Beeston *et al.*, 1935]. The results of the repetition of the work, in which use was made of this finding, are recorded here.

EXPERIMENTAL.

Six groups, each of ten rats, were fed on a basal diet of alcohol- and ether-extracted caseinogen 5, marmite 5, glucose 45, salt mixture 5 parts, with 1 drop of cod-liver oil per rat every 3 days, together with 40 parts of one of the following fats: butter fat, beef fat, palm oil, coconut oil, olive oil and cod-liver oil. The diets were administered for 14 days, and the animals were then guillotined and the livers removed. The total ether-soluble material was extracted from the

Table I. *Weight records of the animals.*

Group no. Fat in diet	...	1 Butter fat	2 Beef fat	3 Palm oil	4 Coconut oil	5 Olive oil	6 Cod-liver oil
Av. initial body wt. (g.)		185	186	195	186	180	187
Av. final body wt. (g.)		187	186	179	177	177	166
Av. gain or loss (g.)		+2	±0	-16	-9	-3	-21
Food intake (g. per rat per day)		10.0	9.5	8.1	9.5	9.2	6.9

pooled livers and its contents of lecithin, cholesterol, cholesteryl esters and glyceride estimated as described by Channon and Wilkinson [1934]. The data relevant to the animal side of the experiment are recorded in Table I.

Results.

1. *Fatty liver production.* Table II records the percentages of each constituent present in the fresh liver and also the absolute weight of each constituent in the liver of the 100 g. rat. These are arranged in order of decreasing fat content of the livers. This table shows that all the fats have caused fatty livers, although the

Table II. *The liver lipoids.*

(a) Percentage of each constituent (g./100 g. fresh liver).

(b) Weight of each constituent present in the liver of the 100 g. rat (g.).

$$\left(\frac{a \times \text{liver as percentage of body weight}}{100} \right).$$

Group no.	1		2		3		4		5		6	
Dietary fat	Butter fat		Beef fat		Palm oil		Coconut oil		Olive oil		Cod-liver oil	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Lecithin	2.59	0.12	2.57	0.12	2.25	0.10	2.92	0.11	3.02	0.10	3.60	0.13
Cholesterol	0.14	0.01	0.20	0.01	0.25	0.01	0.19	0.01	0.23	0.01	0.28	0.01
Cholesteryl oleate	0.64	0.03	0.56	0.03	0.31	0.01	0.31	0.01	0.85	0.03	0.26	0.01
Glyceride	27.30	1.30	23.70	1.06	23.54	1.05	17.12	0.67	11.47	0.39	3.04	0.11
Total % of liver	30.67		27.03		26.35		20.54		15.57		7.18	
Wt. per liver of 100 g. rat (g.)	—	1.46	—	1.22	1.17		0.80		—	0.53	—	0.26
I.V. of food fat	33		40		49		10		80		145	

intensity of the accumulation in the liver varies. Save for cod-liver oil, in the case of which the livers are only slightly fatty (7.18%), the remaining groups contain total lipoids varying from 15.57%, in the case of olive oil, to 30.67% for butter fat; the very high values for butter fat, beef dripping and palm oil, 30.67, 27.03 and 26.35% respectively, are noteworthy in view of the short period of experiment, 14 days only. The results again demonstrate clearly the effectiveness of the 5% protein diet in fatty liver production with a variety of oils and it seems likely that such variations in the nature and amount of the fatty acids present in the livers may provide a means for further elucidation of the fatty liver problem. The percentages of the individual liver lipoids call for little comment. Those of lecithin fall with increasing fat content and the absolute weight of this substance in the liver of the 100 g. rat varies over a roughly similar range. The free cholesterol content varies little from the normal value, 8 mg. in the liver of the 100 g. rat. Both the percentages and absolute weights of the cholesteryl esters show a definite increase, the normal figure being of the order of 0.01%.

As was to be anticipated, the differences in the degree of lipid accumulation in the livers are entirely due to the differing glyceride contents, which vary from 3.04% in the case of cod-liver oil to 27.30% in that of butter. Thus the livers of the animals receiving cod-liver oil contained 0.11 g., three times the normal amount, whilst those of animals receiving butter gave the highest figure and contained 1.303 g., about forty times the normal. Best [1934] remarks that he had found beef fat more effective in producing fatty livers than butter, and the present result is contrary to this finding. Best however used butter, whilst in our experiments filtered butter fat was used and it is possible that the explanation of the different finding lies here. Whilst any caseinogen present in the butter would tend to exercise a lipotropic effect, its amount would be too small

significantly to affect the result. In other studies our colleague, Dr Loach, has demonstrated the presence in milk of choline compounds other than phosphatides. It is possible that these may accumulate in the butter and exert a lipotropic effect, yet be removed in the preparation of butter fat by filtration.

The figures in Table II show that in general the intensity of the fat infiltration of the liver rises inversely to the iodine values of the dietary fat. Thus cod-liver oil, the most unsaturated fat fed in this experiment, I.V. 145, has caused only a very small increase in the glyceride content of the liver, whilst butter fat, I.V. 33, beef fat, I.V. 40 and palm oil, I.V. 49 have resulted in very large increases in the glyceride content of the livers, such increases being however of about the same order. Olive oil, with an intermediate I.V., gives a glyceride content between these two extreme values. Coconut oil is the one exception, for with the lowest I.V., 10, it should have caused the greatest accumulation of liver fat, if the degree of unsaturation were the only controlling factor. It is possible however that the short-chain fatty acids, which constitute a very considerable portion of the fatty acids of this oil, are more readily metabolised and do not therefore play so prominent a part in fatty liver production.

In Table III are recorded the average percentage compositions of groups of the fatty acids of the various food fats used in these experiments. The degree of fat infiltration in the liver runs parallel with the percentages of C_{14} - C_{18} saturated

Table III. *Average percentage composition of fatty acids of the various food fats.*

	Saturated fatty acids		Unsaturated fatty acids		Intake of saturated C_{14} - C_{18} acids g. rat/day
	C_{12} or lower	C_{14} - C_{18}	Lower than C_{18}	Higher than C_{18}	
Butter fat	12	52	--	36	2.08
Beef fat	--	52	--	48	1.98
Palm oil	--	48	--	52	1.55
Coconut oil	63	29	--	8	1.11
Olive oil	--	12	--	87	0.44
Cod-liver oil	--	15	--	40	0.41

acids present in the different fats, except in the cases of olive oil and cod-liver oil. The last column of Table III shows the actual daily intake by each animal of saturated C_{14} - C_{18} acids calculated from the food intake. These figures fall into the same order as do the percentages of liver fat, although from the fact that the intakes by the olive oil and cod-liver oil groups are similar, 0.44 and 0.41 g. per day respectively, it might have been anticipated that these two fats would have caused similar degrees of fat accumulation. The actual amounts of glyceride are however 11.47% for olive oil and 3.04% for cod-liver oil. Whilst a certain deduction cannot be made from these results, the amount of C_{14} - C_{18} saturated acids ingested appears to govern in a general manner the accumulation of fat in the liver. Worthy of notice in this connection are the observations that the inclusion of a relatively saturated fat in the diet of depancreatized dogs, receiving controlled insulin administration, considerably shortened the period before the appearance of signs of liver failure [Allen *et al.*, 1924; Hershey and Soskin, 1931]. One further point needs emphasis regarding the liver fat percentage of the cod-liver oil group; in all the other groups the daily food intake varied from 8.1 to 10.0 g. per animal, while in this group it was only 6.9 g. Further, there are many reports of the toxicity of cod-liver oil which has been demonstrated in a variety of ways. These two factors make it necessary to regard the result with this oil with caution.

2. *The fatty acids present in the livers.* Table IV records the i.v. and mol. wt. of the total liver acids obtained by hydrolysis of part of the total ethereal extract and removal of the unsaponifiable matter. The phosphatide and glyceride

Table IV. *Analysis of the total liver fatty acids.*

Group no. Fat in diet	...	1 Butter fat	2 Beef fat	3 Palm oil	4 Coconut oil	5 Olive oil	6 Cod-liver oil
Total fatty acids in liver (g./100 g. liver)		28.23	24.73	24.19	18.57	13.46	5.55
i.v.		83.5	85.9	87.9	67.9	91.5	158.2
Mol. wt.		270	275	274	257	280	287
Fatty acids of fat fed. i.v.		35	42	51	12	84	150
Mol. wt.		245	276	266	210	280	290

Table V. *Analysis of the liver glyceride fatty acids.*

Group no. Fat in diet	...	1 Butter fat	2 Beef fat	3 Palm oil	4 Coconut oil	5 Olive oil	6 Cod-liver oil
(Glyceride fatty acids (g./100 g. liver))		26.25	22.72	22.16	16.77	12.88	3.31
i.v.		77	74	87	60	83	173
Mol. wt.		271	270	281	261	284	291
Twitchell separation.							
Liquid fatty acids %		62.2	66.3	68.1	57.7	78.1	76.1
i.v.		116	106	121	101	109	176
Mol. wt.		296	300	298	266	289	313
Solid fatty acids %		37.8	33.7	31.9	42.3	21.9	23.9
i.v.		—	14	5	7	10	49
Mol. wt.		—	267	269	257	267	310

Table VI. *Analysis of the liver phosphatide fatty acids.*

Group no. Fat in diet	...	1 Butter fat	2 Beef fat	3 Palm oil	4 Coconut oil	5 Olive oil	6 Cod-liver oil
Phosphatide fatty acids (g./100 g. liver)		1.65	1.59	1.42	1.52	2.11	2.63
i.v.		138	134	149	132	148	143
Mol. wt.		295	296	288	276	291	337
Twitchell separation.							
Liquid fatty acids %		58.3	60.5	59.6	57.3	57.8	57.6
i.v.		185	176	205	189	179	192
Mol. wt.		368	369	350	334	380	350
Solid fatty acids %		41.7	39.5	40.4	42.7	42.2	42.4
i.v.		15	16	21	20	28	15
Mol. wt.		299	289	273	264	319	343

fractions of the total ethereal extracts of the livers were then obtained by means of acetone precipitation. The fatty acids were prepared from each fraction in the usual way and the liquid and solid fatty acids separated by a modified Twitchell process [Hilditch and Priestman, 1931]. The results of these analyses are set out in Tables V and VI.

DISCUSSION.

(a) *Total fatty acids.* From Table IV it is seen that where a greatly increased deposition of fat in the liver has occurred, i.e. in all but the cod-liver oil group, the i.v. of the total fatty acids have decreased quite considerably from the normal (about 115), although the i.v. and mol. wt. of the dietary fats are well reflected in the corresponding values of the liver fatty acids. This is seen particularly well in groups 4, 5 and 6, receiving coconut, olive and cod-liver oils respectively.

(b) *Glyceride fatty acids.* With the exception of the cod-liver oil group, i.v. of the glyceride fatty acids given in Table V are considerably lower than that of the glyceride normally present in the liver (i.v. about 90). These liver glyceride acids reflect in general the nature of the acids fed. Thus those of group 4 have the lowest i.v., 60, and the lowest mol. wt., 261 (coconut oil fatty acids have i.v. 8; mol. wt. 257). With these may be contrasted those from groups 5 and 6. The liver glyceride acids of group 5 have i.v. 83 and mol. wt. 284 (olive oil fatty acids have i.v. 84; mol. wt. 280). Those of group 6 are i.v. 173 and mol. wt. 291 (cod-liver oil fatty acids have i.v. 150; mol. wt. 290). Consideration of the results with these three fats, which provide the best contrast because of their chemical nature, indicates that the composition of the glyceride fatty acids accumulating in the liver is markedly influenced by the dietary fat. Similarly the highest percentages of unsaturated acids are found in the olive and cod-liver oil groups, 78.1 and 76.1 respectively, while the lowest occurs with coconut oil, 57.7. The mol. wts. of these three groups of acids, 289, 313 and 266, also reflect those of the acids of the fats fed. It is noteworthy that apart from the cod-liver oil group the i.v. of the unsaturated acids vary only from 106 to 121, in spite of the varying characteristics of the fats administered. Attention is particularly directed to the i.v. of the acids from the coconut oil group, 101, which differs little from that of the olive oil group, 109, in spite of the fact that coconut oil contains but a very small proportion of unsaturated acids (about 8% of oleic acid, i.v. 90), whilst olive oil (i.v. 83) contains some 87% of C_{18} unsaturated acids. The mol. wt. of the coconut oil group acids, 266, together with the i.v., 101, indicates the presence of considerable amounts of unsaturated acid of less than eighteen carbon atoms and may be evidence of the liver having desaturated the saturated acids of this oil. It is of interest also that there has not been a greater deposition of saturated acids in groups 1, 2 and 3, for it has already been pointed out that the intake of higher saturated acids seems to govern the intensity of the fat accumulation.

(c) *The phosphatide fatty acids.* The i.v. of the phosphatide fatty acids vary only from 132 to 149 and support the finding of Sinclair [1931] that the i.v. of the fatty acids of the food fat and that of the liver phosphatide fatty acids do not run parallel. The i.v. of the liver phosphatide fatty acids from group 4 is also similar to results obtained by Sinclair [1932] when coconut oil was fed. There is however a very considerable difference between the result obtained in group 6 (cod-liver oil) and those obtained by Sinclair [1931; 1932]. That author recorded considerable increases in the i.v. of the liver phosphatide fatty acids up to 170–180, when feeding cod-liver oil in small amounts and up to 21.3% of the diet. His values were much higher than those obtained after coconut and olive oil feeding. In this experiment 40% of cod-liver oil in the diet has been fed, yet the i.v. of the phosphatide fatty acids has increased only to 143, which is not as great as that due to feeding olive oil and only slightly higher than that due to the inclusion of coconut oil in the diet. Further the i.v. of the acids of the glyceride fraction, 173, is considerably higher than that of the phosphatide acids. No

explanation for this divergence can be given. Although the i.v. of the phosphatide fatty acids does not bear any relationship to the i.v. of the food fatty acids, the mol. wts. of these fatty acids do appear to reflect to some extent those of the food fatty acids, save in the case of butter fat, this indicating that the composition of the liver phosphatides may vary to some extent with the dietary fat. Consideration also shows that little difference occurs in the percentages of the liquid and solid fatty acids present in the different groups, the range for the unsaturated acids being 57.3 to 60.5. There is also little variation in the i.v. of the liquid fatty acids, which are considerably higher than those of the glyceride fatty acids, save in the case of group 6 which are only slightly raised. The mol. wts. of the liquid fatty acids are extremely high and fall within a very narrow range. It is possible that these values are inaccurate, since a considerable period of time unavoidably elapsed before the completion of the analyses and chemical change may have occurred, even though the i.v. have remained at a high level. The mol. wts. of the solid fatty acids, although higher than those of the glyceride fatty acids, again reflect to some extent the mol. wts. of the food fatty acids.

These results thus appear to show that the fat occurring in the fatty liver when different fats are fed, if not derived exclusively from the dietary fat, is considerably modified by its nature, which also governs the degree of accumulation occurring. Further discussion of these results, from which many points emerge, will be deferred until work at present in progress has been completed. After removal of the livers and gastro-intestinal tracts of all the animals, the carcasses were pooled and the total fatty acids of each group were worked up. These are being analysed by the ester distillation method after being submitted to the Twitchell process. Discussion of the liver results will be further facilitated when the composition of the depot fats is thus made available. Further, the results at present reported made it appear worth while to put large groups of animals on to the diet used in this paper, with coconut and olive oils, in order to obtain sufficient liver fatty acids for ester distillation, since light on the liver desaturation process, in which choline may be involved, as well as more accurate information concerning the nature of the fatty acids accumulating in the fatty liver under different dietary conditions, will be so obtained.

SUMMARY.

1. Groups of rats have been fed on the diet previously found effective in producing fatty livers and containing 5% caseinogen with 40% fat. Various fats were used and the effectiveness of this diet is shown by the fact that the livers of all the groups were very fatty.

2. The total lipoids present in the livers corresponding to the various fats at the end of 14 days were: butter fat 30.67, beef fat 27.05, palm oil 26.35, coconut oil 20.54, olive oil 15.57, cod-liver oil 7.18% of the fresh liver weight.

3. Factors influencing the variations in the intensity of fat deposition in the livers with the different fats are discussed.

4. The total lipoids were fractionated into phosphatide and glyceride fractions. The fatty acids from all the fractions were analysed by the Twitchell procedure and the i.v. and mol. wt. determined. The results indicated that the nature of the fat in the livers was markedly influenced, both in the glyceride and phosphatide fractions by that of the dietary fat from which much of it was derived.

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CL. FURTHER OBSERVATIONS ON THE EFFECT OF DIETARY CASEINOGEN IN THE PREVENTION OF FATTY LIVERS.¹

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IN one experiment of a series in which study was made of the effect of choline in preventing the accumulation and accelerating the removal of fat in the liver of the rat, Best and Huntsman [1935] observed that, when rats which already had a liver fat percentage of 10–13.5 were transferred to a diet of pure sucrose, the liver fat was further increased by some 8% during the course of 6 days. In a comparable experiment in which the transfer was made to a diet containing 80% of sucrose with 20% of caseinogen, this increase in liver fat did not occur. Assuming a daily food intake of 10 g. per animal, this experiment suggested that 2 g. of caseinogen had prevented an 8% rise in the fat of a liver which was already loaded with fat. Best and Huntsman were unable to decide at that time whether this action of caseinogen was due to the fraction of a mg. of choline present in the diet and they pointed out in further possible explanation of this result that caseinogen might give rise to betaines during its metabolism, since betaine itself was shown to be lipotropically active by Best and Huntsman [1932]. At the same time Channon and Wilkinson [1935] were investigating the production of fatty livers in normal animals on synthetic diets, because they had failed to obtain such livers by the use of the diet of mixed grain and 40% fat originally used by the Toronto workers. Various considerations discussed by them suggested that the amount of the protein present in the diet might be a governing factor in fatty liver production and they concluded from a series of experiments that the amount of fat appearing in the liver was controlled by the amount of protein in the diet, irrespective, however, of any action of choline. In a criticism of these results, Best *et al.* [1935] drew attention to their experiment mentioned above and stated that the results of their more recent unpublished experiments suggested that 1 g. of the caseinogen used by them had no greater effect on liver fat than did 0.5 mg. of choline. They further stated that, whilst the lipotropic effect of caseinogen was not to be underestimated, in their opinion the effect could be accounted for if the protein contained 0.2 or 0.3% of choline, betaine or other substances with a similar action.

Meanwhile Beeston *et al.* [1935] had applied their previous findings on this protein effect to a further extensive study of the "cholesterol" fatty liver with results which amply confirmed their original conclusion that the caseinogen present in the diet controlled the amount of glyceride appearing in the liver, irrespective of any action of choline. As typical of this powerful action of the caseinogen may be mentioned an experiment in which the amount of glyceride in the livers of a group of animals was reduced by 18.69% by the extra daily

¹ The substance of this paper was reported at a Meeting of the Biochemical Society, on 15th Nov. 1935 [Beeston and Channon, 1935].

intake of only 1 g. of protein per rat. Such figures as these left no doubt as to the validity of the conclusion that traces of contaminating choline could not be responsible for the effect and personal discussion of these and unpublished Toronto results led to Best and Channon [1935] stating that contaminating choline could account for only an insignificant part of the caseinogen effect.

A further point which emerged was that the lipotropic effect of the caseinogen used in Liverpool appeared to be very much greater than of that used in Toronto, for in their experiment mentioned above, Best and Huntsman found that 2 g. prevented an 8% rise in liver fat, whilst in this laboratory, in preventive experiments on normal animals both with the "fat" fatty liver and the "cholesterol" fatty liver, a very much greater effect had been repeatedly observed.

Apart from emphasising this considerable difference both in the degree of the lipotropic action of caseinogen in the results from the two laboratories and in its interpretation, Best *et al.* [1935] noted that in certain experiments the percentages of fat observed in the Toronto laboratories were higher than those obtained here. A constant amount of marmite was used in the different diets of all our groups of animals as a source of B-vitamins and Best *et al.* suggested that marmite might contain other unknown lipotropic factors which would lower the liver fat percentages. They further stated that the 2 mg. of choline received by our rats might be a significant factor in the conditions of our experiments.

The work described in this paper was therefore carried out with three objects in view. Firstly it was clearly desirable to express the preventive action of caseinogen on liver fat in terms of a standard, choline, for only by such means can the lipotropic actions of different proteins be accurately compared; secondly to investigate the effect of marmite on the degree of fat accumulation in the liver; and thirdly to determine the minimum amount of choline which exercised an effect on liver fat in the diet containing 5% caseinogen and 40% fat generally used by us. It was hoped that these experiments, the results of which have already appeared in summarised form [Beeston and Channon, 1935] would further establish or finally dispose of the apparent disparity between the results at Toronto and those at Liverpool.

EXPERIMENTAL.

The method of experiment was to feed ten groups of rats, each of 8–10 animals, on a basal diet consisting of caseinogen (alcohol- and ether-extracted) 5, fat (beef dripping) 40, glucose 45, salt mixture 5, cod-liver oil 1 part. Five of the groups received in addition 5 parts of marmite, whilst in the remaining five this was substituted by a further 5 parts of glucose. In each of the two series varying percentages of choline chloride were added to the diet. Two further groups of animals received, with and without marmite, a diet differing from the basal diet in containing 30 parts of caseinogen, 25 parts of the glucose of the basal diet being substituted by this protein. After 14 days the animals were killed by guillotine and the amount of fatty acids and unsaponifiable matter in the livers determined by the methods previously used [Channon and Wilkinson, 1935]. In Table I are recorded the essential data regarding the animal side of the experiment. Choline determinations on the food constituents were made by the method of hydrolysis followed by acetylation and biological assay on the isolated rabbit intestine. The marmite used in these experiments contained 440 mg. of choline per 100 g. and the caseinogen 0.6 mg. per 100 g. The daily food consumption was measured and in the figures in Table II the total daily choline intake includes not only the added choline in groups 1 *b*-1 *e*, and 2 *b*-2 *c*, but in series 1 that

Table I.

Diet		1. Marmite present				2. Marmite absent			
		No. of animals	Av. final wt. g.	Av. wt. change \pm % initial body wt. g.	Food consumption g./rat/day	No. of animals	Av. final wt. g.	Av. wt. change \pm % initial body wt. g.	Food consumption g./rat/day
Caseinogen %	Choline chloride %								
(a)	5 + 0.00	9	156	- 0.1	8.2	8	146	- 8.7	7.8
(b)	5 + 0.02	8	169	- 3.0	8.7	8	156	- 5.2	8.2
(c)	5 + 0.05	9	167	0.4	9.1	9	152	7.0	7.3
(d)	5 + 0.10	9	162	+ 3.3	9.2	7	152	- 5.7	7.5
(e)	5 + 0.20	9	167	+ 2.3	8.6	8	160	- 3.2	7.7
(f)	30 + 0.00	9	178	+ 10.0	8.6	9	164	+ 1.0	7.3

Table II.

	Diet		1. Marmite present			2. Marmite absent		
			Daily intake			Daily intake		
	Pro- tein %	Choline chloride %	Total choline mg./rat	Casein ogen g./rat	Liver fat % wet wt.	Total choline mg./rat	Casein- ogen g./rat	Liver fat % wet wt.
(a)	5	+0.00	1.8	0.41	20.45	0.0	0.39	23.60
(b)	5	+0.02	3.4	0.43	13.14	1.4	0.41	24.33
(c)	5	+0.05	5.9	0.45	9.62	3.2	0.36	12.44
(d)	5	+0.10	10.0	0.46	8.58	6.5	0.38	8.32
(e)	5	+0.20	16.8	0.43	6.20	13.3	0.38	6.74
(f)	30	+0.00	1.9	2.58	5.62	0.0	2.20	6.84

present also in the marmite provided in the diet. It is to be mentioned that the groups 1f and 2f in Table II in which about 2 g. more caseinogen were ingested than in groups a-e, received in consequence 0.01 mg. additional choline per rat per day, an amount which is insignificant and which may be disregarded in the discussion.

The lipotropic action of caseinogen in reference to choline as standard.

The figures in Table II show that in all the groups a-e in series 1 and 2 the caseinogen intake per rat per day varied between 0.36 and 0.46 g., a figure which may be taken as sufficiently constant to permit a satisfactory comparison of the different results. In group 1a the caseinogen intake of 0.41 g. per rat per day has yielded a liver fat percentage of 20.45. In the same series in group 1f a caseinogen intake of 2.58 g. per rat per day has yielded a liver fat percentage of 5.62. Thus an extra intake of 2.17 g. of caseinogen per rat per day has lowered the liver fat percentage by 14.83. Further, in series 2, in which marmite was absent, an extra intake of 1.81 g. of protein has lowered the liver fat from 23.6 to 6.84%, a reduction of 16.76%. These figures correspond to reductions of 7 and 9% in the liver fat respectively per g. of protein ingested. The lowest figures for the liver fat in the two series caused by the greatest amounts of choline given, 16.8 and 13.3 mg. per rat per day respectively, are 6.20 and 6.74% (groups 1e and 2e). If these be taken as the same as those resulting from the 30% protein diets in groups 1f and 2f, 5.62 and 6.84% respectively, then in series 1, 2.17 g. of protein have had an effect equivalent to 16.8 mg. of choline, while in series 2, 1.81 g. of protein have had an effect equivalent to 13.3 mg. of choline. Hence in series 1, 1 g. of protein has yielded an effect on the percentage of liver fat equivalent to 7.7 mg. of choline, while in series 2 it is equivalent to 7.3 mg. of choline.

With these figures is to be contrasted the statement of Best *et al.* [1935] that in unpublished results following their experiment mentioned above 1 g. of caseinogen is equivalent in action to not more than 0.5 mg. of choline, which is about one-fifteenth of the value recorded here.

In experiments which have since been carried out on normal animals by the preventive technique originally used by us in investigating this problem, Best, Grant and Ridout [1936] have now fully substantiated our findings [Channon and Wilkinson, 1935; Beeston *et al.*, 1935] that caseinogen exercises a very considerable action on liver fat in this type of experiment. They now emphasise the importance of limiting the caseinogen content of the diet, if the rapid production of large fatty livers is desired, and it is for this reason that we have adopted during the last two years the use of a diet containing 5 % of caseinogen and 40 % of fat [Channon and Wilkinson, 1935; Beeston *et al.*, 1935; Channon and Smith, 1936]. It is of considerable interest also that in this type of experiment Best, Grant and Ridout [1936] find that 1 g. of their caseinogen exercises an effect comparable with "perhaps 5 or 6 mg. of choline". This confirmation of our results [Beeston and Channon, 1935] by the Toronto workers makes it very unlikely that there is any difference in the lipotropic values of the caseinogen preparations used in the two laboratories, for the difference between their figure of 5-6 mg. and ours of 7-8 mg. is, in our opinion, well within the range of experimental error. As these authors point out, it thus appears that the lipotropic action of caseinogen in the transfer experiment on which they originally based their criticism of the results of Channon and Wilkinson [1935; Best, Huntsman and Ridout, 1935], is far less than in the preventive type of experiment carried out on normal animals. If the active substance present in caseinogen is a contaminant, the possibility exists that its amount may vary in different preparations of the protein. It is therefore of interest that the Toronto workers have now obtained a result similar to our own, when employing the same experimental method, even though feeding caseinogen from a different source.

Best, Grant and Ridout [1936] also confirm that the fat content of the liver decreases to approximately normal value as the protein content of the diet is increased from 5 to 30 % and make the additional observation that further increase in the dietary protein to 50 % causes no further lowering of the liver fat percentage. With their figure for a liver fat percentage of 5.04 on the 30 % protein diet, which they mention as being only slightly above normal, may be compared the corresponding figures in Table II, which are 5.62 and 6.84 % respectively. These figures also are definitely above the normal value, which is usually taken as 3-4 %. In all our experiments of this type we have never obtained a figure as low as 4 %, although, as is discussed later, neither has choline in the amounts used by us caused a reduction to the normal figure.

It is not known at the present time whether caseinogen effects this lipotropic action through some integral part of its molecule or by virtue of some contaminating substance. Various possibilities have been discussed [Best and Huntsman, 1935; Channon and Wilkinson, 1935; Best and Channon, 1935; Beeston, Channon and Wilkinson, 1935; Best, Mawson, McHenry and Ridout, 1936]. We would like briefly to draw attention to one further possibility. The extensive experiments of Best and Huntsman [1935] showed that when animals, whether with normal livers or livers already loaded with fat, were transferred to a diet of pure sucrose only, the amount of fat in the liver was markedly increased and the authors commented on the possibility of a "specific" effect of sucrose. These results, together with the fact that the mixed grain diet with 40 % of fat used by the Toronto workers in their original work, although providing some 10 mg. of

choline daily for each animal, yet caused fatty livers, led us to enquire whether this effect of increasing protein intake might be due merely to the corresponding decrease in carbohydrate intake. We endeavoured to investigate this possibility by feeding three groups of animals on diets containing 20 % of fat, 2 % of cholesterol and 5 % of caseinogen; one group served as control, whilst in the second and third groups 5 % and 25 % of glycine were added at the expense of the corresponding amounts of glucose of the basal diet. The object of this experiment on the cholesterol fatty liver was to cut down the carbohydrate intake by substitution of a pure amino-acid which might be expected to have no lipotropic effect. This experiment was carried on for 21 days. The percentages of total ether-soluble material in the pooled livers of these three groups were: control 39.4 %; 5 % glycine 42.4 %; 25 % glycine 34.4 %. We feel that the somewhat lower figure of 34.4 % on the diet with 25 % glycine is of doubtful significance, since the food consumption on this particular diet was not good and the weight losses considerable (21.7 % of the initial body weight). Hence these results appear to dispose of the idea that "dilution" of the carbohydrate is the cause of the protein effect. Some figures recorded by Best, Grant and Ridout [1936] in an experiment designed for another purpose appear to provide confirmation of this. They fed three groups of animals on a diet containing 40 % of fat with (a) 10 % caseinogen, (b) 30 % caseinogen and (c) 10 % caseinogen with 20 % gelatin. While the increase of the caseinogen content from 10 to 30 % reduced the fat content of the liver from 24.7 to 6.5 %, the addition of 20 % of gelatin to the diet containing 10 % caseinogen had no effect, the recorded figure, 22.4 %, not being significantly different.

Apart from this interesting observation that gelatin appears to possess no lipotropic activity, Best, Grant and Ridout [1936] report that dried egg white possesses an activity approximately equal to that of caseinogen, whilst minced ox muscle proteins have a smaller activity than that of caseinogen. In this laboratory studies of the lipotropic actions of different proteins are also in progress. For this purpose we have prepared a number of plant and animal proteins of varying biological value and these are now being tested against caseinogen, with and without the addition of certain essential amino-acids. It will not be possible to express their activities in terms of caseinogen with accuracy, because of the difficulty of preparing pure proteins in sufficient amount, since 1-2 kg. of each protein are required for a single experiment on two groups of animals. At the present stage we can only report that edestin appears to exercise a considerable lipotropic action, since groups of animals receiving a diet containing 40 % of fat and 5 % of edestin yielded a liver fat of 23.7 %, while those receiving 25 % of edestin and 40 % of fat gave a figure of 9.0 %. The further results will be reported in due course.

The effect of marmite on the liver fat percentage.

The effect of marmite on the liver fat percentages can be seen from the figures in Table II, in which are recorded the choline intakes of the two series of animals, together with the percentages of liver fat resulting. Considering first series 2, in which marmite was absent, it is seen that in group 2a, with a choline intake of zero, a liver fat percentage of 23.6 % resulted. The intake of 1.43 mg., group 2b, has had no effect on the liver fat percentage, the difference between the resulting figure, 24.33 %, and that of group 2a, 23.6 %, being of no significance in view of the normal group variations. Increase of the choline intake to 3.2 mg., group 2c, has caused a marked effect in lowering the liver fat to 12 % and the percentage progressively decreases through groups 2d and 2e to 6.74 %

with increasing choline intake. In the series in which marmite was present, group 1 *a*, in which the choline intake was 1.8 mg., yielded a liver fat of 20.45 %, a figure which is little different from that obtained in group 2 *b* (marmite absent, choline intake 1.4 mg.). The increase of the choline intake to 3.4 mg., group 1 *b*, has also caused a sharp fall in the liver fat percentage to 13.14 %, a figure again of a very similar order to that occurring in group 2 *b*, 12.44 %, in which the choline intake was of the same order, 3.2 mg. A further increase to 5.9 mg. causes a further fall to 9.62 %, group 1 *c*, which again is quite similar to the figure in group 2 *d* at a similar choline intake. Similar remarks apply to group 1 *d* compared with group 2 *e*.

It appears therefore from these figures that the presence or absence of marmite in the diets has had no effect on the liver fat except by virtue of the choline which it contains and that no other lipotropic substances are present in sufficient amount to cause any obvious effect. These results therefore appear to discount the suggestion made by Best, Huntsman and Ridout [1935] that the lack of greater deposition of liver fat in our experimental animals could be attributed to lipotropic substances other than choline contained in the marmite. On the other hand, although marmite has exercised no lipotropic effect other than that due to its contained choline, it is not unlikely that this lack of effect is merely the balance between the opposing factors of substances tending to increase liver fat and those tending to decrease it. Thus McHenry [1936] has shown that vitamin B₁ increases the amount of fat in the liver. Whilst the results of series 1 do not show any increase over those of series 2, in spite of the vitamin B₁ provided as marmite, this is probably explained by the fact that only on diets of extremely low choline content was the effect of vitamin B₁ observed [McHenry, 1936]. Further it is probable that a yeast extract such as marmite will contain cystine, of which amounts as low as 7.5 mg. per rat per day have been found to be markedly effective in increasing the percentage of liver fat on this particular diet [Beeston and Channon, 1936]. Any effect of these two factors tending to increase liver fat may be offset by betaines, the presence of which is probable in such an extract.

The minimum effective dose of choline.

The figures in Table II show very clearly that about 3 mg. of choline have caused a marked effect in decreasing the liver fat percentage. Thus in groups 1 *a* and 2 *b*, in which the choline intake was 1–2 mg., the percentages do not differ from those of 2 *a*, in which no choline was ingested. On the other hand in groups 1 *b* and 2 *c*, in which the choline intake has been raised to 3.4 and 3.2 mg. respectively, a sharp fall in the liver fat percentages to 13.4 and 12.44 % respectively has resulted. These figures thus show that on the diet most frequently used in this laboratory, containing 40 % of fat and 5 % of protein, 3 mg. of choline have a marked preventive effect. It is interesting to note that Best, Mawson, McHenry and Ridout [1936] have obtained a similar figure on a diet containing 15 % of protein and 40 % of fat. On the other hand it is seen in groups 1 *e* and 2 *e*, in which 16.8 and 13.3 mg. of choline were administered per rat per day, that the liver fat percentages are 6.2 and 6.74 % respectively, figures which are definitely above normal. We have repeatedly encountered this finding that choline in the amounts used by us has not reduced the liver fat to normal on this particular diet. Thus in the experiments of Channon and Smith [1936] in which the effect of triethyl- β -hydroxyethylammonium hydroxide was compared with that of choline, a large number of groups of animals was used. All these groups received the 40 % fat and 5 % protein diet for periods varying from 13 to

20 days and in addition received choline in amounts varying from 8.8 to 79.8 mg. per rat per day. If all the results be pooled, the mean value for the fat content of the livers of these 64 animals was 7.75 %, a figure approaching twice the normal, while the lowest figure obtained for any group was 5.2 % and in 5 out of 11 groups a figure of 8 % or over was obtained. It thus appears that, whilst small amounts of choline have a very marked effect in preventing a rise in the liver fat percentage, a considerably greater quantity is necessary to maintain it at a normal level.

SUMMARY.

1. In continuance of previous studies of the effect of dietary protein in preventing the occurrence of fatty livers in rats receiving diets containing 40 % of fat, the action of caseinogen has been equated against that of choline. 1 g. of caseinogen is equivalent in its preventive action on liver fat deposition to 7–8 mg. of choline.

2. The provision of marmite, or its absence from the diet, had no further effect on liver fat accumulation than that anticipated from its choline content.

3. In preventive experiments on diets containing 5 % of caseinogen and 40 % of fat, 3 mg. of choline per rat per day caused the liver fat percentage to be reduced from 20 to 10 %. On the other hand it is pointed out that amounts varying from 8.8 to 79.8 mg. a day did not prevent some fat accumulation occurring in the liver.

4. Edestin is shown to have a lipotropic action.

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CLI. A RAPID METHOD FOR THE ESTIMATION OF TOTAL IRON IN BLOOD.

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Of the current methods for the determination of total iron in blood, none appears to be entirely quantitative [Burmester, 1934]. The method here described however has been found reasonably accurate and is well suited to routine analysis where numerous samples must be examined in a relatively short time.

The blood is digested with a sulphuric-nitric acid mixture and the Fe estimated by a modification of Hill's [1931] $\alpha\alpha'$ -dipyridyl method. The detailed procedure is as follows. Pipette 0.5 ml. of oxalated blood into a pyrex test-tube (12 x 2 cm.). Add 1.5 ml. of Fe-free concentrated H_2SO_4 and 0.5 ml. of redistilled HNO_3 . Heat cautiously over a micro-burner, with continuous shaking, until nitrous fumes cease to be evolved. Cool slightly, add 2 drops HNO_3 and heat as before, repeating until a clear solution is obtained and the nitrous fumes are completely expelled. Cool and make up to 25 ml. with distilled water. Mix 1 ml. of this solution with 1 ml. of $\alpha\alpha'$ -dipyridyl reagent (0.0012 M $\alpha\alpha'$ -dipyridyl in 5 N Fe-free ammonium acetate) in a 1 cm. tube of a Lovibond tintometer. Add $\text{Na}_2\text{S}_2\text{O}_4$ grain by grain with continuous shaking until the maximum colour is developed (only a trace is needed; a large excess would produce cloudy solutions). The intensity of the red colour is then almost directly proportional to the Fe content within the concentration limits observed (Fig. 1). The Fe content may therefore be determined either from the approximate relation: mg. Fe/100 ml. = 6.44 (Lovibond red units), or from a graph constructed by treating a series of standard Fe solutions exactly as described above. Fig. 1 was obtained in this manner (except that the red colour was measured in a 1 cm. round cell of a Rosenheim-Schuster tintometer).

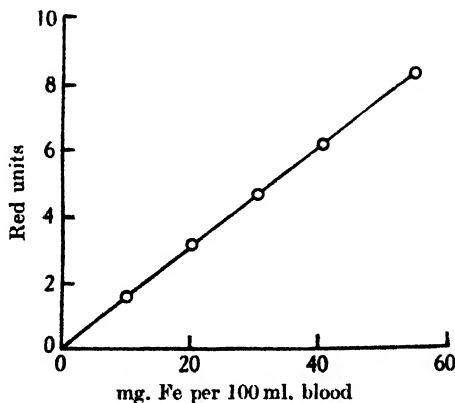


Fig. 1.

In Table I are presented the results of an analysis of oxalated sheep blood by the present method and by the usual thiocyanate procedure as described by Peters and Van Slyke [1932].

Table I.

mg. Fe per 100 ml. blood	
Present method	Thiocyanate method
43·5	45·5
42·0	41·5
40·5	42·0
35·0	35·0
33·5	33·5
27·5	27·0
26·0	26·5

The authors wish to express their thanks and appreciation to Mr B. C. Aston, of this laboratory, for his kind interest in this work.

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CLII. THE ACTION OF CERTAIN REAGENTS ON THE "LOOSELY BOUND" IRON IN BLOOD.

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STUDIES of the iron in vertebrate blood have shown that the iron of the haemoglobin molecule is to be distinguished from the residual iron, which may reside partly in the corpuscles and partly in the plasma [see, especially, Barkan, 1925; 1927; 1933]. This important distinction has been established by at least three independent methods, supposedly giving similar values for non-haemoglobin iron. These are: (1) Colorimetric estimation of non-haemoglobin iron on an ultrafiltrate from blood which had been incubated with dilute hydrochloric acid [Barkan, 1925; 1927; 1933]. (2) Iodimetric estimation of iron in the trichloroacetic acid filtrate from blood which had been boiled with 5N hydrochloric acid [Starkenstein and Weden, 1928]. (3) Colorimetric estimation of iron in the trichloroacetic acid filtrate from blood which had been treated with thiolacetic acid [Tompsett, 1934, 1].

For whole blood or serum, Tompsett [1934, 1] has shown that 20% trichloroacetic acid does not completely extract iron, added as iron alum, except in the presence of thiolacetic acid. Quantitative recovery of added inorganic iron in presence of thiolacetic acid however is not sufficient proof of the suitability of this reagent for the determination of non-haemoglobin iron in blood. Shorland and Wall [1936, 1] have shown in preliminary work that thiolacetic acid can liberate iron from haemoglobin, and this conclusion is now substantiated in this paper.

We have therefore examined sodium pyrophosphate [Tompsett, 1934, 2; 1935] as a possible alternative reagent and compared the results with those given by the Starkenstein and Weden [1928] method.

EXPERIMENTAL.

Experiments were made on samples of oxalated sheep's blood. Filter-papers were washed with dil. HCl and distilled water to remove traces of Fe and then dried. The trichloroacetic acid solution was prepared from freshly distilled acid. Periodic blank experiments showed the reagents to be practically free from Fe.

(1) *The action of thiolacetic acid on haemoglobin iron.*

In these experiments a method essentially similar to that recommended by Tompsett [1934, 1] was followed. 2 ml. of blood were diluted with an equal volume of water and treated with a specified quantity of thiolacetic acid. 4 ml. of 20% trichloroacetic acid were immediately stirred in and the solution filtered after a given time through a no. 41 Whatman filter-paper. 1 ml. of filtrate was treated with 2 drops of conc. ammonia in a 1 ml. cup of a Dubosecq colorimeter. The standard colour was prepared similarly from 2 ml. of a stock solution of

iron alum containing 2 mg. Fe in 100 ml. 1% H_2SO_4 . Comparison is made after dilution of the unknown to approximately the same depth of colour as the standard and the non-haemoglobin Fe evaluated.

Fig. 1 shows the variation of apparent non-haemoglobin Fe with time at two concentrations of thiolacetic acid. Fig. 2 shows the effect of varying the concentration of thiolacetic acid from 2 to 25 drops (1 drop = 0.05 ml. approx.) in a 48-hour reaction.

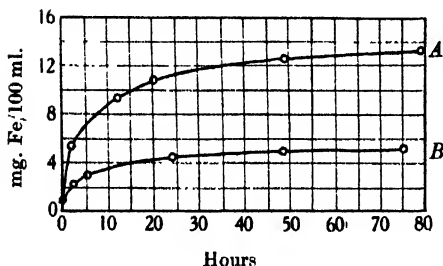


Fig. 1.

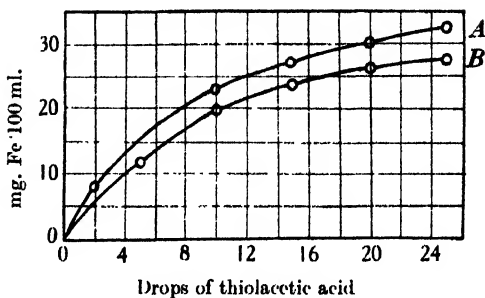


Fig. 2.

Fig. 1. Effect of time on the value obtained for non-haemoglobin iron by thiolacetic acid. Total Fe, 45 mg./100 ml. (A) 5 drops thiolacetic acid; (B) 1 drop thiolacetic acid.

Fig. 2. Effect of concentration of thiolacetic acid on the liberation of iron from blood. (A) Total Fe, 45 mg./100 ml. (B) Total Fe, 34 mg./100 ml.

Clearly, the time taken for the estimation, which is not specified by Tompsett [1934, 1], would materially affect the apparent value for the non-haemoglobin Fe. When the period of contact was short (3 min.), values of 0.83 to 1.09 mg./100 ml. were obtained for non-haemoglobin Fe. These compare with 0.96 to 1.56 mg./100 ml. for human blood [Tompsett, 1934, 1].

(2) *Sodium pyrophosphate as a reagent for the determination of non-haemoglobin iron.*

Method. Mix 2 ml. of oxalated blood and 2 ml. of 4% sodium pyrophosphate in a test-tube. After 15 min. stir in 2 ml. of 20% trichloroacetic acid solution and filter through a no. 41 Whatman paper. Add 2 ml. of filtrate to 1 ml. of 0.0012 *M* $\alpha\alpha'$ -dipyridyl in 5 *M* ammonium acetate. After developing the colour as described in Shorland and Wall's [1936, 2] modification of Hill's method [1931], compare with standard colour (prepared similarly from 2 ml. of a solution of iron alum containing 1 mg. Fe/100 ml. 1% H_2SO_4) in a Duboseq colorimeter.

Table I. *The determination by the sodium pyrophosphate method of inorganic iron added to blood.*

mg. Fe/100 ml.		
Non-haemoglobin Fe of sample	Added inorganic Fe	Reported non-haemoglobin Fe
0.72	1.00	1.70
0.33*	1.00	1.33
0.38*	1.00	1.35
0.87	2.00	2.86
0.86	2.00	2.85
0.51	2.00	2.51

* Determinations made on samples which had stood several days.

Table I shows that inorganic Fe, added as iron alum, is quantitatively recovered, affording an indication of the efficiency of the method. It is not, however, certain that non-haemoglobin Fe is fully determined by this reagent, as Barkan [1933] has shown that there is in blood the "easily-separable" Fe which can be distinguished from inorganic Fe by its failure to be adsorbed on aluminium hydroxide. Tompsett [1934, 2] has suggested the use of pyrophosphate for the liberation of Fe from such complexes.

The action of sodium pyrophosphate on the haemoglobin Fe was tested by allowing the reagent to stand in contact with blood for different periods prior to estimation of the liberated Fe (Table II).

Table II. *The effect of time of contact of the sodium pyrophosphate solution with blood on the reported non-haemoglobin iron.*

Time of contact (min.)	Reported non-haemoglobin Fe (mg. Fe/100 ml.)
Sample (A) 10	0.83
20	0.83
Sample (B) 5	0.70
20	0.70
50	0.70

Table III shows that fresh blood gives a higher non-haemoglobin Fe value by this method than blood which has stood several days

Table III. *The effect of ageing of blood samples on their reported non-haemoglobin iron content.*

		mg. Fe/100 ml			
Fresh blood	3 days old	Fresh	8 days old	Fresh	15 days old
0.71	0.30	0.86	0.38	0.91	0.59
0.81	0.29	0.68	0.27	0.80	0.56
0.89	0.33	0.59	0.33	0.75	0.48
0.86	0.32	0.77	0.61	—	—
0.96	0.42	0.71	0.57	—	—

The actual rate of change of non-haemoglobin Fe as given by this method is shown for a particular sample as below:

Table IV.

Hours	mg. Fe/100 ml.
0	0.89
24	0.80
36	0.51
48	0.43

Barkan [1927] observed that by use of his method the apparent non-haemoglobin Fe increased if the blood were aged for several weeks prior to the determination. The reverse seems to be true of the present method.

Although, so far as the authors are aware, comparable figures for the non-haemoglobin Fe of sheep's blood are not available, Barkan [1927] finds for several species of vertebrates, including man, a mean value of approximately 1.7 mg. Fe per 100 ml. For human blood this has been confirmed by Starkenstein and Weden [1928], whilst Tompsett [1934, 1] gives lower values varying from 0.96 to 1.56 mg. Fe per 100 ml.

Even for fresh blood, the values given by the sodium pyrophosphate method appear to be somewhat low. The method was therefore compared (Table V)

with that of Starkenstein and Weden [1928]. Firstly, however, the statement of Starkenstein and Weden that boiling 5*N* HCl had no appreciable action on haemoglobin Fe was confirmed: boiling the sample for 1 min. with the 5*N* acid resulted in practically the same figure for non-haemoglobin Fe as boiling for 5 min.

Table V. *Reported non-haemoglobin iron.*

Mg./100 ml.	
Sodium pyrophosphate method	Starkenstein and Weden's method
0.79	1.22
0.86	1.52
0.76	1.69
0.71	1.25
0.87	2.00
0.89	1.73
0.97	1.79
0.89	1.64
0.80	1.88

The values for the non-haemoglobin Fe obtained by the sodium pyrophosphate method are much lower than those obtained with Starkenstein and Weden's reagent. Since both these reagents are without effect on the haemoglobin Fe under the experimental conditions used it seems that part of the non-haemoglobin Fe must be more labile than the rest.

SUMMARY.

The actions of thiolacetic acid and sodium pyrophosphate respectively on the iron in blood have been examined. The former reagent has a definite action on haemoglobin iron and is therefore not suitable for the estimation of non-haemoglobin iron. Good recoveries of inorganic iron added to blood are given by the latter reagent which is shown not to attack haemoglobin iron. The values obtained for the non-haemoglobin iron by the sodium pyrophosphate method, however, appear to be considerably lower than those obtained by means of Starkenstein and Weden's reagent (boiling 5*N* hydrochloric acid).

In conclusion the authors wish to express their appreciation to Mr B. C. Aston, of this Department, for the opportunity of carrying out this investigation.

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CLIII. THE B-VITAMINS IN HUMAN URINE.

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IN recent years much attention has been directed to the part played by lack of the B-vitamins in the production of a number of diseases of obscure origin, and the use of one or more constituents of the vitamin B complex has been advocated for the cure of a variety of conditions. A review of this subject will be found in the "Discussion on avitaminosis (B group) in tropical and temperate countries" at the Royal Society of Medicine, in 1934.

In particular, the occurrence in a number of diseases of a multiple peripheral neuritis, similar to that of beriberi, has suggested a causal relationship of the B-vitamins. Cobb and Coggeshall [1934] have summarised the causes of neuritis and given a list of conditions in which it occurs as the possible result of dietary deficiency; among these are included combined system disease, occurring in pernicious anaemia [Gildea *et al.*, 1930], and "alcoholic" polyneuritis [Minot *et al.*, 1933; Strauss, 1935]. The deficiency in the former state has been thought possibly to be due in some cases to defective absorption of the vitamins, in the latter state to an insufficient intake, since advanced alcoholic addicts consume alcohol at the expense of more normal, vitamin-containing constituents of the diet.

Unfortunately, most of the evidence connecting the B-vitamins with these conditions has been of a curative nature, and as such does not throw much light on their causes, since the fact that a substance cures a disease does not necessarily prove that its lack has caused the disease. A further disadvantage of human curative observations of a dietetic nature is the difficulty of ensuring that only one constituent of the diet is altered at a time. This is particularly true when additional vitamin B₁ is fed, since it has been shown [Cowgill *et al.*, 1925] that this increases the appetite, so that there is a concurrent increase in the intake of all other constituents of the diet. But perhaps the most serious disadvantage till recently has been that no pure sources of the B-vitamins have been available for clinical use, so that therapeutic effects obtained from feeding yeast or even liver have often been attributed solely to the B-vitamins contained in these substances. However, as the knowledge of the composition of the various B-vitamins advances, it is becoming possible to investigate their separate effects. Some therapeutic effects of crystalline vitamin B₁ have already been examined [Vorhaus *et al.*, 1935].

The assumption that conditions such as "alcoholic polyneuritis" and combined system disease are caused by lack of the B-vitamins has therefore rested on the fact that they occur in patients who have, it is thought, been living on diets deficient in the vitamins. It is, however, seldom easy to decide whether a diet has contained adequate B-vitamins; for the distribution of the B-vitamins in

¹ Working with a Rockefeller Fellowship for Medical Research, awarded by the Medical Research Council.

foods is widespread and variable, the quantities consumed are always difficult to ascertain, the amounts needed by man have not been definitely settled, and there is no certainty that the amount consumed will always be absorbed.

A method of discovering whether a patient is in a deficient condition is therefore badly needed. By adopting the method used with vitamin C [Harris *et al.*, 1933; Johnson and Zilva, 1934], a study was made of the vitamin B₁ and B₂ content of human urine, in the hope that the amount of these vitamins excreted might give some indication of a surplus or deficiency of them in the body. The urine of three normal subjects was examined and also that of a patient with "alcoholic" polyneuritis.

HISTORICAL.

The presence of an antineuritic factor in human and animal urine was shown by Muckenfuss [1918], Gaglio [1919] and Van de Walle [1922], the last two also showing that the amount was reduced in starvation and in vitamin B₁ deficiency. More recently, Gulick and Daniels [1931] investigated the vitamin B (B₁) content of the milk feeds, urine and faeces of eleven infants. They found a fairly uniform retention of vitamin B, increasing with the size of the child.

Helmer [1935] investigated the amount of vitamins B₁ and B₂ in urine from three normal subjects receiving a weighed, well balanced diet and in the urine from one patient with untreated pellagra. He found that whilst the urine from the normal subjects contained appreciable amounts of both vitamins B₁ and B₂, neither of these vitamins could be detected in the urine from the pellagrin.

Thus there are already indications that the amount of the B-vitamins in the urine is affected by the oral intake of vitamins, but it is not known whether any quantitative relationship exists.

EXPERIMENTAL.

1. The subjects studied and their vitamin intake.

A study was made of the excretion of vitamins B₁ and B₂ in three control male subjects (I, II and IV), admitted to hospital for reasons unconnected with diet or metabolism, and in one male patient (III) with "alcoholic" polyneuritis.

This patient, 53 years of age, had for years consumed moderate amounts of alcohol, at times as much as 1 pint of whisky a day but usually not more than 2 or 3 pints a week. For years he had eaten irregularly, sometimes taking practically nothing but 1 pint of whisky daily, during several days, as his apparent source of calories. It was impossible to determine any regular dietary habits. Recently his appetite had been very poor. There had been no prominent gastro-intestinal symptoms but there was marked decrease of hydrochloric acid in the stomach contents.

A month before entry he noted tenderness of the calves of the legs, sensations as of pins and needles in the feet. At that time progressive "weakness" of the legs began. Examination on entry to the hospital showed definite but not severe signs of "alcoholic" polyneuritis. Strength was diminished in the legs and feet. The gait was ataxic on a slightly broad base. Knee jerks were diminished and ankle jerks were absent. Babinski's sign was not present. The calves were markedly tender on pressure. There was slight hyperaesthesia of the soles of the feet. Vibration sense was moderately diminished, but position sense was apparently normal. By the end of the observations, which included a period (3) during which a high vitamin diet was fed, diminished ankle jerks were the only residual sign.

Since it was not possible to feed diets of known vitamin content, the excretions at different levels of vitamin intake were compared. In each case the subjects received the different diets for 12 days and the vitamins in the total

urine excreted during this period were estimated. By this procedure it was hoped to eliminate variations caused by day to day changes in the food and by any lag in excretion when the diet was changed.

The control "normal" subjects were observed for two successive periods of increasing vitamin intake, periods 1 and 2 below. The patient with "alcoholic" polyneuritis was observed for a third period, 3, with a still higher vitamin intake. This patient received 1 quart of whisky daily throughout the experiment. The diets given were as follows:

Period 1. The ordinary hospital diet.

Period 2. The same diet with daily additions of 15 g. (10 tablets) of "Harris Yeast Vitamin" and 18 g. (3 level teaspoonfuls) of "Vegex" ("Marmite").

Period 3. A high vitamin diet as used for the treatment of cases of "alcoholic" polyneuritis by Minot *et al.* [1933] and by Strauss [1935], together with "Harris Yeast Vitamin" and "Vegex" in the same amounts as in Period 2.

"Harris Yeast Vitamin" (12.5 g.) and "Vegex" (15 g.) in the proportions fed to the patients in Periods 2 and 3, were mixed with water, made up to 100 ml. with water, and the B-vitamins estimated in this preparation, which will be referred to as the "B-vitamin mixture". The amount of additional vitamins fed in Period 2, as compared with Period 1, was thus roughly ascertained.

The vitamin excretion was estimated in the urine only. The amount excreted in the faeces was not taken into account, since it would almost certainly be affected by bacterial action in the gastro-intestinal tract and therefore be no guide to the true excretion. At the same time it must be remembered that, especially when large amounts of the vitamins are being fed, it is possible that absorption may be incomplete, so that any balance sheet constructed without consideration of exogenous excretion of the vitamins can only be approximate.

The total urine for each 12-day period was collected, 10% of 95% alcohol containing 3% of HCl, being used as preservative. It was then concentrated at about 50° *in vacuo* to a volume of 1 litre. The concentration could be carried to this point without appreciable sedimentation occurring. The concentrated material was brought to a p_H of about 4.0 with NaOH and stored in the cold till used. 83 ml. of the final preparation then represented a day's output of urine.

It has recently been shown that vitamin B₂ can be resolved into two factors [Kuhn *et al.*, 1933, 1, 2, *etc.*], one factor being a lyochrome (flavin) and the second known as supplement or vitamin B₆. In this work, however, the vitamin B complex was estimated as a whole, so that there is no indication whether the urines tested would not, on further work, be found to contain large amounts of either flavin or vitamin B₆. That they did contain both these factors to some extent was however demonstrated, for in the absence of either factor, no "vitamin B₂ complex" activity could have been observed by the growth method employed.

2. Details of the vitamin tests employed.

Vitamin B₁ estimations. The method of estimating this vitamin was a modification of that described by Burn [1932] and Coward *et al.* [1933]. Determination was made of the percentage of animals, in the original work pigeons, in the present study rats, which were cured of the neuritis which develops, when vitamin B₁ is absent from the diet, after a given dose of vitamin B₁-containing material. A curve showing the percentage of animals cured by different amounts of a standard vitamin B₁ preparation was constructed at the same time, and by

comparison with this, the amount of the vitamin in the tested dose of each material could be found.

This curative method was employed for two reasons. Since only one dose of vitamin-containing material was used for each animal, the total quantity needed for a test was small, whereas in growth tests doses are fed daily over a period of weeks to each animal. Also, difficulty was experienced in persuading animals deprived of vitamin B₁ to consume daily doses of concentrated urine, whereas a single dose could be introduced artificially.

The rats used weighed 100–200 g. and had previously received a synthetic ration with adequate, but not superabundant, B-vitamins. They were given a diet containing vitamin B₂ but no vitamin B₁, this being constituted as follows: washed caseinogen (R. H. Thomas) 20 g.; corn starch 60 g.; salt mixture (McCollum No. 61) [Itter *et al.*, 1935] 5 g.; and "Crisco" 15 g.; mixed with 100 ml. of a watery solution of autoclaved "Vegex" (1 hour at p_H 9.0) containing the equivalent of 50 g. of "Vegex", to supply vitamin B₂. The diet was cooked for 2–3 hours in a steam steriliser. Cod liver oil was fed as a separate daily dose.

After 3 or more weeks, during which time the rats lost about one-third of their weight, acute neuritis developed. Three degrees of severity of symptoms were recognised, slight, definite and severe, and rats with each degree were used to titrate each dose, but no correlation was found between ease of cure and the severity of symptoms. Similarly it was not found that the weight of the rats when dosed influenced the cures.

As soon as the nervous symptoms were apparent, a single dose of the material to be tested was introduced into the stomach, by means of a pump made from a graduated 5 ml. syringe to which was attached a length of small size rubber catheter. In this way it was possible to ensure that the animal received the whole of its dose. The rat was then observed for 2 days, at the end of which time the effect of the dose was always clear.

The results of the tests are shown in Table 1. The curative effects of the following materials were investigated.

Table I. *The curative effect of various vitamin B₁-containing materials on rat neuritis.*

Material		Doses tested (ml.) and number of rats cured. In brackets the number of rats observed						Amounts needed to cure 50–70 % of animals
Urines*	Subject	Period	0.06	0.125	0.25	0.5	1.0	
I	(1)	—	—	0 (1)	0 (1)	0 (1)	0 (1)	> 2.0 ml.
	(2)	—	—	0 (1)	0 (2)	4 (6)	—	0.5 ml.
II	(1)	—	—	—	—	—	0 (1)	> 2.0 ml.
	(2)	—	—	0 (2)	4 (6)	—	—	0.5 ml.
IV	(1)	—	—	—	—	0 (3)	—	> 1.0 ml.
	(2)	—	—	—	0 (4)	3 (6)	—	1.0 ml.
III	(1)	—	—	—	—	0 (1)	0 (1)	> 2.0 ml.
	(2)	—	0 (2)	3 (6)	—	—	—	0.25 ml.
	(3)	0 (2)	3 (6)	—	—	—	—	0.125 ml.
B-vitamin mixture (ml.)			0.125	0.25	0.5	0.25–0.5 ml.		
			0 (1)	0 (4)	6 (7)			
Acid clay, international standard (mg.)			10	20	30	20 mg.		
			3 (10)	6 (10)	8 (10)			

* Concentrated so that 1 litre \equiv 12 days' output.

(a) *Standard.* With the international standard acid clay adsorbate of vitamin B₁, and with 10 rats receiving each dose, 10 mg. (1 International Unit) produced cures in 30 % of the animals, 20 mg. (2 I.U.) in 60 % and 30 mg. (3 I.U.) in 80 %.

The curve expressing these results is shown in Fig. 1, together with the curve given by Coward *et al.* [1933] for pigeons. It will be seen that the two are exactly

comparable in form, but that the pigeon appears to need twice as much vitamin B₁ as the rat. Thus to cure 50 % of the pigeons a dose of 30 mg. was required, while apparently 15 mg. would have been sufficient for rats; and to cure 80 % of the pigeons 60 mg. were required, while for rats only 30 mg. were needed.

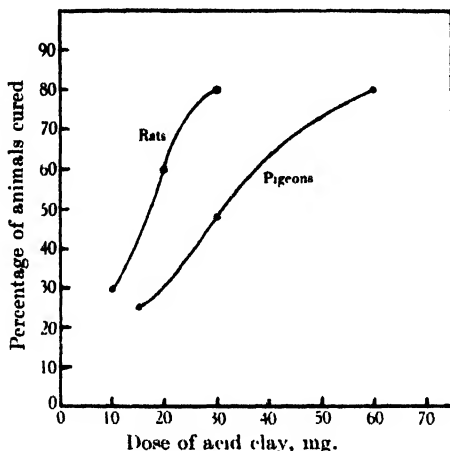


Fig. 1. Response to graded doses of acid clay adsorbate (international vitamin B₁ standard) of pigeons and rats with neuritis due to vitamin B₁ deficiency.

This result contradicts that obtained by Kinnersley *et al.* [1930], who found that the vitamin B₁ requirements of pigeons and rats were equal, as judged by the number of days after a single dose of test material during which cures of neuritis lasted. It agrees, however, with the statement made by Plimmer *et al.* [1927], that whilst pigeons need 4 % dry yeast in the diet to maintain weight, rats only need 2 %. It is probable that the different amounts of vitamin B₁ needed by the pigeon and the rat are related to the different body weights of these species, thus the pigeon may be considered to be roughly twice as large as the rat. This matter has been investigated fully by Cowgill [1932], who took restoration of appetite in depleted animals as the criterion of vitamin B₁ activity. At the same time it may be pointed out that Cowgill's other hypothesis, that the need of animals of a given species for the vitamin increases with increasing weight, was not supported in these observations, where cure of neuritis was the criterion, for the weight of the rats, within the limits observed, 88–179 g., did not influence the cures.

(b) *Urines*. When testing the urines it was found that if the doses tested were of the order 1, 2, 4, 8 *etc.*, the minimum dose which had any effect cured 50–60 % of the rats. Such minimum effective doses, irrespective of the exact proportion of the animals cured, were taken to contain equal quantities of vitamin B₁ and to be equivalent to 20 mg. of acid clay, which promoted cures in 60 % of the animals, that is equivalent to 2 i.u. No doubt, if the number of animals observed with doses of half these amounts had been larger, a small proportion would have been cured, comparable with the proportion, 30 %, cured by 10 mg. of acid clay.

It will be seen that no vitamin B₁ could be detected in the urine from patients receiving the ordinary hospital diet without additional vitamins. Period 1, although as much as 2.0 ml. of the concentrated urine was tested. Larger amounts than this could not be given as they appeared to be toxic, causing the death of the animals before the effect on the nervous symptoms could

be observed. Whether these urines did in fact contain small amounts of vitamin B₁ remains therefore in doubt, and it can only be said that 2 ml. of concentrated urine contained less than 2 I.U. of vitamin B₁, so that 83 ml. concentrated urine, or 1 day's output, contained less than 83 I.U. The urines from the patients receiving additional B-vitamins, Periods 2 and 3, all contained appreciable amounts of vitamin B₁, from 0.125 to 2.0 ml. containing 2 I.U., or, 1 day's output (83 ml.) containing 167–1333 I.U. of vitamin B₁.

(c) *B-vitamin mixture, given to subjects during Periods 2 and 3.* The minimum amount of this material found to cure (0.5 ml.) relieved the symptoms of 86 % of the rats observed; 0.5 ml. would thus seem to be equivalent in effect to 30 mg. of acid clay (80 % cures) and so to contain 3 I.U. of vitamin B₁. It was therefore calculated that $\frac{0.5}{3}$ ml., that is 0.17 ml. contained 1 I.U. of vitamin B₁. It would have been expected that some of the animals receiving 0.25 ml. (1.5 I.U.) would have been cured; actually none of the 4 rats observed was cured.

The equivalent of 120 ml. of this B-vitamin mixture was fed daily to the patients during Periods 2 and 3 of the experiments. They therefore received 720 I.U. of vitamin B₁ in addition to the amount present in their food.

Vitamin B₂ estimations. The growth method of Chick and Roscoe [1928] was employed for the estimation of this vitamin.

Rats of 35–40 g. weight were given a diet consisting of washed caseinogen (R. H. Thomas) 20 g.; corn starch 60 g.; salt mixture (McCollum No. 51) [Itter *et al.*, 1935] 5 g.; and "Crisco" 15 g.; mixed with 100 ml. of water and cooked for 2–3 hours in a steam steriliser. After 1 week the rats were also given 0.1 ml. daily of a vitamin B₁ concentrate, prepared from brewer's yeast [Kinnersley *et al.*, 1933], an amount equivalent to 0.6 g. dry weight of the original yeast and containing sufficient vitamin B₁ for normal growth. Cod liver oil was fed as a separate daily dose. The animals were observed for a further week or until they were no longer growing, and the doses of the vitamin B₂ containing materials were then fed. The feeding of these materials was continued for 5 weeks, 6 days a week, and the growth during this period observed. In order to increase the palatability, sugar was mixed with the doses of urine.

In Table II are shown the results obtained when the vitamin B₂ in the B-vitamin mixture and in the concentrated urines was tested by this method.

Table II. *The growth promoting effect of various vitamin B₂-containing materials for rats.*

		Amounts of urine* and B-vitamin mixture fed to young rats (40–60 g. weight) as source of vitamin B ₂ , together with the resulting weight increase in 5 weeks. In brackets the number of rats observed							
Material		Average weight increase g.	Dose ml.	Average weight increase g.	Dose ml.	Average weight increase g.	Dose ml.	Average weight increase g.	
Urines*	Subject Period								
I	(1)	0.5	45 (4)	1.0	59 (4)	—	—	—	—
	(2)	—	—	—	—	0.25	62 (6)	0.5	67 (2)
II	(1)	0.25	43 (4)	0.5	57 (2)	—	—	—	—
	(2)	—	—	0.25	56 (6)	—	—	—	—
IV	(1)	—	—	0.125	56 (4)	0.25	59 (5)	0.5	64 (1)
	(2)	—	—	—	—	0.125	61 (4)	0.25	58 (5)
III	(1)	—	—	0.25	56 (4)	0.5	64 (2)	—	—
	(2)	—	—	0.25	53 (4)	0.5	61 (2)	—	—
	(3)	—	—	0.06	50 (4)	0.125	63 (5)	0.25	68 (3)
B-vitamin mixture	—	—	—	0.125	56 (4)	0.25	61 (4)	—	—

* Concentrated so that 1 litre = 12 days' output.

It will be seen that large variations in the amount of vitamin B₂-containing materials fed produced only small differences in the growth response. Thus the concentrated urine from subject IV, during Period 1, was tested in three different doses, 0.125, 0.25 and 0.5 ml. and the average growth during 5 weeks observed in rats receiving these amounts was 56, 59, and 64 g. This result was entirely different from the results obtained previously with this method, when rats bred at the Lister Institute, London, were used. As an example of these results the test for vitamin B₂ in dried banana may be quoted [Roscoe, 1931, Table III]; 1 g. of banana promoted 35 g. growth in 5 weeks, 2 g. promoted 62 and 4 g. promoted 102 g.

It was at first thought that the inability of large amounts of the concentrated urines to stimulate a greater weight increase might be due to a toxic material in the urines which inhibited growth. Two facts were opposed to this theory however, the first being that growth did not appear to be affected by the actual size of the dose, irrespective of the vitamin content, and the second that when other rats received the B-vitamin mixture as source of vitamin B₂, the difference between the responses to 0.125 and 0.25 ml. was also very slight, the average weight increase with these two doses being 56 and 61 g. respectively. It seems, therefore, that for some unexplained reason, the rats did not respond to graded doses of vitamin B₂ in the same way as those employed in previous experiments.

This fact emphasises the necessity for employing in all vitamin B₂ tests some material as standard with which others may be compared, and of not relying on a standard growth response. Only in this way can comparisons be made between the results obtained in different laboratories, for it is apparent that growth response is an uncertain factor, varying according to the strain of animals employed, and even, it is possible, in the same strain according to the time of year.

The absence of a marked difference in weight increase as a result of feeding widely different amounts of vitamin B₂ considerably reduced the accuracy of the tests, but by comparing two or three different doses of each material it was found possible to assess their relative vitamin B₂ contents. In Table II the doses of the materials tested are arranged so that amounts promoting equal weight increases fall in line. The amounts in each column may then be considered to have contained equal quantities of vitamin B₂.

Since the materials tested in this work were not compared with a standard material, it must be understood that no conclusions can be drawn as to the absolute amounts of vitamin B₂ present. But since the B-vitamin mixture, fed to the patients as source of vitamin B₂ and the urines excreted were tested at the same time and by the same method, a rough estimation of the proportion of the additional intake of the vitamin which was excreted could be made. For the purpose of this work, the vitamin B₂ in the urines was expressed in terms of the amount present in 1 ml. of the B-vitamin mixture, this being called 1 ml. equivalent of vitamin B₂. Then since "Vegex" and "Harris Yeast Vitamin" were fed to the subjects, during Periods 2 and 3, in amounts equal to those present in 120 ml. of the B-vitamin mixture, the additional vitamin B₂ intake, during these periods, was 120 ml. equivalents.

From 0.06 to 1.0 ml. of the different concentrated urines was found to be equal in vitamin B₂ content to 0.125 ml. of the B-vitamin mixture, or 0.5-8.0 ml. contained 1 ml. equivalent of the vitamin. Since 83 ml. of concentrated urine represented in each case 1 day's output of urine, the daily output of vitamin B₂ in different urines varied from approximately 160 to 10 ml. equivalents daily.

3. The vitamin B_1 and B_2 contents of the urine of different subjects.

In Table III the intake and urinary output of vitamins B_1 and B_2 are given for the four subjects. The results relating to the two vitamins will be considered separately.

Table III. *Intake and urinary output of vitamins B_1 and B_2 in four subjects.*

Intake of vitamins B_1 and B_2 : All subjects		Periods (12 days each)		
		(1) Hospital diet	(2) Hospital diet + vitamin B_1 720 I.U. and vitamin B_2 120 ml. equivalents* daily	(3) High vitamin diet + vitamin B_1 720 I.U. and vitamin B_2 120 ml. equivalents* daily
Output and retention of vitamin B_1 (I.U. daily):		Output	Output	Increased retention
I } Controls		< 83	333	387-470
II } Controls		< 83	333	387-470
IV } Controls		< 167	167	553-720
III "Alcoholic" polyneuritis		< 83	667	53-136
Output and retention of vitamin B_2 (ml. equivalents* daily):		Output	Output	Increased retention
I } Controls		10	80	50
II } Controls		20	40	100
IV } Controls		80	160	40
III "Alcoholic" polyneuritis		40	40	120

* Arbitrary units adopted for this work only, see p. 1059.

Vitamin B_1 . As already stated, the amount of this vitamin present in the urine of the subjects receiving the ordinary hospital diet (Period 1) was so small that it could not be estimated by the method here used. This might mean that this diet contained so little of the vitamin that it was all utilised by the patients. The diet was, however, a liberal one, so that this does not seem probable.

The increased intake of vitamin B_1 during Period 2 was reflected in an increased excretion in the case of all the control subjects (I, II and IV). The amounts excreted in the urine were 333, 333 and 167 I.U. daily. Assuming that the vitamin B_1 in the hospital diet remained constant, so that the additional intake during this period was that provided by the "Harris Yeast Vitamin" and the "Vegex", that is 720 I.U. daily, the increased retention during Period 2 could be calculated. It is shown in Table III, and will be seen to have been between 387 and 470 I.U. daily in two cases and between 553 and 720 I.U. in one case. Thus an increased consumption of vitamin B_1 resulted in increased excretion and increased retention.

It was expected that patient III with "alcoholic" polyneuritis, who, it was thought, had been suffering from a deficiency of vitamin B_1 , might, when the intake was high, retain more of the ingested vitamin than the other subjects, to replenish depleted stores. This was not so. During Period 1, again no vitamin B_1 was detected in the urine, so no comparison could be made. During Period 2, the patient actually excreted more vitamin B_1 (667 I.U. daily) than did any of the controls (maximum 333 I.U. daily), and retained less (increased retention of between 53 and 136 I.U. daily). During Period 3, the output of this vitamin was

still further increased, 1333 i.u. daily being excreted, or twice as much as during Period 2.

This large excretion of vitamin B₁, by a patient who was receiving alcohol daily throughout the experiment, might seem to support the theory put forward by Gigon and Odermatt [1925], that consumption of alcohol results in a washing of this vitamin out of the body, by way of the urine. However, since only one case was observed, no definite conclusions can be drawn.

Vitamin B₂. The normal subjects (I, II and IV) all excreted definite, though small, amounts of vitamin B₂ in the urine during the period when they received the hospital diet alone (Period 1). The amounts excreted were 10, 20 and 80 ml. equivalents daily. When, in Period 2, the intake was increased by 120 ml. equivalents daily, the output was also increased, being 80, 40 and 160 ml. equivalents daily. The increased retention, assuming a constantly increased consumption of 120 ml. equivalents daily, was then 50, 100 and 40 ml. equivalents daily. Thus, as for vitamin B₁, there was an increase in both excretion and retention following an increased intake. The proportion of the additional vitamin excreted to that retained was very variable, being 70 : 50, 20 : 100 and 80 : 40.

Subject III with "alcoholic" neuritis, excreted 40 ml. equivalents of vitamin B₂ daily during Period 1, an amount not different from that excreted by the controls. However, when the intake was increased by 120 ml. equivalents (Period 2), the excretion was not increased, remaining at 40 ml. equivalents daily. The patient therefore appeared to have retained the additional 120 ml. equivalents of vitamin B₂, suggesting that there had previously been a deficiency. The retention of one of the controls was however, nearly as large: thus Subject II retained 100 ml. equivalents daily. It is therefore doubtful whether the retention can be considered to have been significantly large. When the intake of Subject III was increased still more (Period 3), an increased excretion was observed, 160 ml. equivalents daily, as compared with 40 ml. equivalents daily in Periods 1 and 2.

DISCUSSION.

The absolute amount of either vitamin B₁ or vitamin B₂ present in the urine at a given time would not appear to give any clear indication of the adequacy of the diet, for the subjects, even when receiving an adequate diet, excreted only small amounts of these vitamins. It even appeared that vitamin B₁ might be absent from the urines of subjects receiving a normal mixed diet, though it is more probable, especially in view of previous work on the subject, that some was excreted, but that the quantities were too small to be appreciated by the method used. Thus the maximum amount of concentrated urine that it was found possible to test was 2 ml. or 1/40 of a day's output, whereas Helmer [1935], using urine evaporated to dryness, was able to test doses representing 1/25 of a day's output, and found vitamin B₁ activity in such.

Since considerable variations in urinary output of vitamins B₁ and B₂ were exhibited by different "normal" subjects receiving the same diet, no mean normal excretion on a given diet could be found. This result may, however, have been due in part to variations in the amount of the diets consumed, which, in these experiments, was not controlled.

When the intake of vitamins B₁ and B₂ by control "normal" subjects was increased by a known amount, some of the additional vitamin was excreted in the urine and some retained or disposed of by other means. The amount retained was variable, both in absolute amount and in proportion to the amount excreted.

Such variations occurring in normal subjects showed that extreme caution must be exercised in interpreting the results obtained with patients who are supposedly deficient. The absolute amount excreted would not appear to be significant, and any variations in the retention of the vitamins, from that found in normals, would have to be very large to be considered abnormal. Thus it is doubtful whether the slight variations observed in the one patient with "alcoholic" polyneuritis studied in this work, a decreased retention of vitamin B₁ and an increased retention of vitamin B₂ when additional vitamins were fed, can be considered to have been significant.

The results obtained do not on the whole encourage the belief that the balance sheet method can be applied to the study of the metabolism of the B-vitamins, though this question cannot be finally settled until more extensive experiments are carried out with diets of known vitamin content, and until it can be ensured that all the vitamin administered is absorbed.

SUMMARY.

1. Vitamins B₁ and B₂ were estimated in the urine of four subjects, three normal controls and one case of "alcoholic" polyneuritis. The subjects first received the hospital diet for 12 days, and then, for a further 12 days, the same diet with a known daily addition of B-vitamins. The case of "alcoholic" polyneuritis also received a special high vitamin diet with additional B-vitamins for a third period of 12 days.

2. The total urine excreted during these periods was concentrated and fed to rats deprived of vitamin B₁ or B₂ respectively. The vitamin B₁ activity was estimated by the power of the urines to cure neuritis, the vitamin B₂ activity by their power to promote growth.

3. This method of estimating vitamin B₁, which consisted of finding the proportion of a number of rats that was cured when single doses of the test material were fed, has not previously been used with rats as test animals. The method was standardised by observing the effects obtained with the international standard acid clay adsorbate of vitamin B₁.

4. Vitamin B₁ was not found in the urine of control subjects receiving the hospital diet, though it is probable that small amounts were present. The addition of 720 I.U. of vitamin B₁ daily to this diet was followed by the daily excretion of 167-333 I.U. in the urine, the remainder of the additional intake may be assumed to have been retained.

5. Vitamin B₂ was present in small quantities in the urine of control subjects receiving the hospital diet. Feeding additional vitamin B₂ increased the amount excreted in the urine and at the same time the retention appeared to have been increased.

6. In the one case of "alcoholic" polyneuritis studied, the initial excretion of vitamins B₁ and B₂ was comparable with that of the controls. When additional B-vitamins were fed, the retention of vitamin B₁ was slightly less than that observed in any of the controls, and the retention of vitamin B₂ slightly greater. The differences were, however, probably too small to be significant.

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CLIV. METABOLISM OF NORMAL AND TUMOUR TISSUES.

XVI. ACTION OF SOME OXIDATION-REDUCTION SYSTEMS.

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THE action of reversibly oxidisable dyestuffs on the metabolism of living animal cells was described in important experiments of Harrop and Barron [1928] and Barron and Harrop [1928]. Barron [1929] examined the action on respiration of erythrocytes and Barron and Hoffman [1930] on that of starfish eggs; they were able to show a rough correlation with the oxidation potential of the dyes. When the rate of reduction of the dye by metabolites and of oxidation of the leuco-dye by atmospheric oxygen proceeded more rapidly than the normal oxidation processes in the cell, the rate of cellular oxidation was increased on addition of the dye.

Corresponding with the increased oxidations in erythrocytes, they found a decreased aerobic glycolysis, and the effect of the dyes was also roughly proportional to the fermentative power of the cell. This latter effect was found by Barron [1930] to extend to slices of mammalian tissues, using methylene blue as dyestuff: Barron did not study the effect of methylene blue on aerobic glycolysis in these tissues. The greatest effect of methylene blue in increasing oxidation is shown with tumour, since tumour tissue has a very high glycolysis. This has been confirmed by Jares [1935], who showed also that the R.Q. of tumour tissue was little affected by methylene blue: methylene blue therefore does not restore the defective carbohydrate oxidation of tumour tissue [Dickens and Šimer, 1930.]

Elliott and Baker [1935] have also studied the action of some oxidation-reduction dyes on normal and tumour tissues, mainly with a view to explaining the inhibition of kidney respiration by 2:6-dichlorophenolindophenol.

The toxicity of many of these dyestuffs is often considerable and may cause an increase of metabolism to be completely masked except in the more favourable cases. Several dyestuffs have been found to exert a greater effect on respiration than does methylene blue, and this is partly attributable to the greater toxicity of methylene blue, a factor considered earlier by Quastel and Wheatley [1931] and also emphasised by Friedheim [1934], who showed that pyocyanine is less toxic and causes a great increase in respiration accompanied by a variable but often large inhibition of the aerobic glycolysis of tumours.

The experiments to be described show that certain other dyes which greatly accelerate oxidation, though apparently no more poisonous than pyocyanine, do not show any similar effect on aerobic glycolysis. On the other hand some substances closely related chemically to pyocyanine have similar effects in

lowering aerobic glycolysis and increasing oxidation of tumour tissues. Since pyocyanine is found also to inhibit anaerobic glycolysis, its action on glycolysis appears not to depend merely upon the respiratory increase which it produces. Moreover, measurements of R.Q. show that the defective carbohydrate oxidation of tumour is not restored by pyocyanine, so that it cannot be regarded as transforming the metabolism of tumour more nearly to normal by remedying some fundamental deficiency of the enzyme systems of tumour tissue.

EXPERIMENTAL.

Methods and reagents.

Respiration, aerobic and anaerobic glycolysis and respiratory quotient were measured on tissue slices suspended in Ringer solution by the methods of Warburg and by those previously fully described in this series. The medium was buffered with phosphate or bicarbonate- CO_2 to p_{H} 7.4 in the usual way; when glucose or lactate was added the concentration was 0.2%. The time of experiment was usually one hour unless stated.

The dyes used were not specially purified but were those sold as oxidation-reduction indicators. Bindschedler's green (B.D.H.) and Capri blue were obtained as zinc salts and were freed from zinc before use by H_2S and extraction of the leuco-base; in the case of Bindschedler's green oxidation of the latter is slow and the solution was oxygenated for several hours before use. Capri blue was prepared by the method of Cohen and Preisler [1931]: 6:7-dimethylalloxazine according to Kuhn and Rudy [1934]. Sources of the other materials are acknowledged at the end of this paper. Since several of these substances are sparingly soluble in salt solution, the solutions were prepared by very thorough grinding in a mortar, but in some cases the dye was present partly as a finely divided suspension; the concentrations given are the total amounts present in the liquid. Figures for E_{h} at p_{H} 7 appearing in the Tables are quoted from Stern [1934].

Toxicity: action on kidney metabolism.

With kidney cortex, a tissue with low glycolytic power, toxic effects are more evident than with tissues showing aerobic glycolysis [Barron, 1930; Friedheim, 1934]. The effect of substances used was therefore first studied on respiration of kidney tissue in glucose media as an indication of their relative toxicities. Since different samples of kidney had to be used this comparison is imperfect but nevertheless useful. Results are shown in Table I.

With methylene blue Barron [1930] reported an inhibition of respiration of 14%, Jares [1935] found for 3×10^{-4} M concentration a transient initial rise, which we have also observed, followed by a fall of 9%. Elliott and Baker [1935] give -62% for 10^{-3} M and -27% for 10^{-5} M . Methylene blue is thus decidedly toxic.

With 2:6-dichlorophenolindophenol (1.3×10^{-3} M) Elliott and Baker [1935] found 92% inhibition, and with Bindschedler's green 10^{-3} M 83%, which agree with our measurements. Bindschedler's green undergoes oxidation during the experiment to indophenol blue [Cohen and Preisler, 1931], with measurable O_2 uptake for which a correction has been applied.

Nile blue is about as toxic as methylene blue, whilst cresyl violet, phenosafranine, Janus green, neutral red and phenazine methiodide are only slightly poisonous in the concentrations used.

Table I. *Respiration of rat kidney (mainly cortex).*

Substance added	E_h at pH 7 (volt)	Medium	% alteration of respiration for dye concentration						
			2.5×10^{-3}	10^{-3}	2×10^{-4}	10^{-4}	6×10^{-5}	3×10^{-5}	$10^{-5} M$
Glucose									
Bindschedler's green	0.224	Phos.	—	-79	—	-24	—	-8	-6
2:6-Dichlorophenolindophenol	0.217	"	-74	—	—	—	—	—	—
Thionine	0.062	"	—	—	+3	—	+12	—	—
					(+28 to -22)		(+28 to +1)		
		Bic.	—	—	+16	—	+31	—	—
					(+29 to +4)		(+40 to +28)		
Brilliant cresyl blue	0.047	Phos.	—	+1	+44	+23	+9	-2	—
		Bic.	—	-8	—	—	+19	—	—
Methylene blue	0.011	Phos.	—	-73	-60	+2	—	-6	—
Nile blue	-0.116	"	—	-58	-37	-37	—	-29	—
Cresyl violet	-0.166	"	—	—	-18	-13	—	-4	—
Phenosafranine	-0.252	"	—	28	-8	-4	—	-3	—
Janus green	-0.256	"	—	-14	+7	+2	—	-5	—
Neutral red	0.330	"	—	-13	+9	+11	—	+11	—
Phenazine-methiodide	—	"	—	—	—	+7	—	+12	—
Lactate.									
Thionine	—	Phos.	—	—	+34	—	+59	+90	+55
	—	Bic.	—	—	—	—	+43	—	—
Brilliant cresyl blue	—	Phos.	—	-8	—	+39	—	+18	+9
	—	Bic.	—	—	—	+35	—	—	—
Galloyaniline	0.021	"	—	—	—	+6	-3	—	—
Methylene blue	—	"	—	—	—	-13	—	—	—
Pyocyanine	-0.034	"	—	—	+15	+23	-12	—	—
	—	"	—	—	+10	—	—	—	—

Thionine (Lauth's violet B.D.H.) and brilliant cresyl blue (B.D.H.) provide the most interesting results in Table I, since in some experiments they caused considerable increase of kidney oxidation; the results were variable with different specimens of kidney (Table II). Pyocyanine causes an increase in phosphate- but not in carbonate-Ringer [Friedheim, 1934]; with thionine and cresyl blue the increase was sometimes shown in phosphate, but a more marked increase occurred in bicarbonate, media, reaching +44 % with cresyl blue.

Table II. *Rat kidney in glucose; thionine and brilliant cresyl blue.*

Substance added	Conc. M	Medium	Q_{O_2}			R.Q.	Control			R.Q.
			1st hr.	2nd hr.	3rd hr.		1st hr.	2nd hr.	3rd hr.	
Thionine	6×10^{-5}	Phos.	-30.3	-21.3	-22.2	—	21.4	-20.7	-19.3	—
	2×10^{-4}	"	-21.2	-14.7	-9.2					
	6×10^{-5}	Bic.	-33.6	-26.2	-17.4					
	2×10^{-4}	"	-32.5	-28.5	-19.6	0.89	-21.7	-17.3	-16.7	—
	6×10^{-5}	Phos.	-24.3	-16.0	—					
							-19.7	-17.8	—	0.85
Brilliant cresyl blue			Q_{O_2}	$Q_{O_2}^{1/2}$	$Q_{O_2}^{2/3}$		Q_{O_2}	$Q_{O_2}^{1/2}$	$Q_{O_2}^{2/3}$	
	6×10^{-5}	Bic. (2 hr.)	-26.5	+1.7	+5.4	—	-20.7	+1.1	+6.2	—
		dupl.	-29.1	-0.2	+4.0	—	-20.7	+1.1	+4.9	—
	10^{-3}	Phos.	-17.4	—	—	—	-17.0	dupl.	—	—
	5×10^{-4}	"	-24.8	—	—					
	5×10^{-5}	"	-20.6	—	—					
	10^{-4}	"	-27.9	—	—	—	-22.3	—	—	—
	5×10^{-5}	"	-21.9	—	—					
	2×10^{-5}	"	-22.1	—	—					
	5×10^{-4}	Bic.	-19.1	—	—	—	-19.7	—	—	—
	5×10^{-5}	"	-29.0	—	—					

Table III. *Rat kidney in lactate; thionine and brilliant cresyl blue.*

Substance added	Concentration <i>M</i>	Medium	Q_{O_2}		R.Q.	Q_{O_2}	Q'_{O_2}	R.Q.
			1st hr.	2nd hr.				
Thionine	2×10^{-5}	Phos.	- 30.4	- 29.6				
	2×10^{-4}	"	- 42.7	- 33.8				
			Q_{O_2}	Q'_{O_2}	R.Q.	Q_{O_2}	Q'_{O_2}	R.Q.
	6×10^{-5}	Bic. dupl.	- 41.7	- 10.1				
			- 43.7	- 11.1	0.95	- 26.9	- 6.1	0.82
	6×10^{-5}	"	- 43.3	- 12.4	0.88	- 28.2	- 6.0	0.85
					0.885	- 31.2	- 8.3	0.815
	5×10^{-4}	Phos.	- 22.8			- 24.3	- 25.5	dupl.
Cresyl blue	10^{-4}	"	- 36.1					
	5×10^{-5}	"	- 27.8					
	10^{-5}	"	- 27.1					
	10^{-4}	Bic.	- 25.4			- 22.4	- 35.5	dupl.
	10^{-4}	"	- 55.2					

The respiration of kidney in lactate is usually considerably higher than in glucose, and in the presence of either of these dyes it is still further strongly increased either in phosphate or bicarbonate media: pyocyanine also caused some increase, whilst methylene blue showed a progressive toxic action and gallocyanine had no effect (Table III). This action of thionine and brilliant cresyl blue is strictly comparable with that of dinitro-*o*-cresol [Dodds and Greville, 1933] which causes an exactly similar effect. These dyes are distinctly poisonous in high concentrations, consequently an optimum concentration exists which seems to depend on some property of the individual piece of tissue: variation of the position of this maximum probably accounts for differences with different specimens. This phenomenon has been frequently observed, the optimum concentration being often a little less than that which is markedly toxic (Tables I and IV).

The R.Q. in glucose may be slightly increased by these two dyes. In lactate a marked increase of R.Q. is shown, and since with thionine the extra CO_2 is approximately equal to the extra O_2 (Table III) it appears that the increase is mainly due to oxidation of lactate or carbohydrate. Corresponding with the increased oxidation of lactate an increase of base occurs in the solution.

Effect on tumour oxidation.

The experimental material was Jensen sarcoma and Walker/256 carcinoma of the rat, and mouse tumour 37S; I am indebted to Dr Cramer of the Imperial Cancer Research Fund Laboratories for these original tumours. The action of a fairly representative series of oxidation-reduction indicators on the metabolism of these tissues is shown in Table IV.

Only one experiment in each of the two media is quoted for each dye and for each tumour, unless great variation in the results occurred.

It appears from Table IV that the maximum action of these indicators in increasing tumour respiration in glucose is exerted over a limited range of oxidation-reduction potential. Thus ferricyanide has little effect as have also the strongly negative systems phenol red, cresyl violet, phenosafranine or benzylviologen. This result is similar to that of Barron and Hoffman [1930] with starfish eggs.

The greatest sustained increase, amounting to nearly +200% in some cases, was obtained with thionine and brilliant cresyl blue, the two dyestuffs found least

Table IV. Action on tumour metabolism.

Substance added	E_h at $P_H 7$ (volt)	Conc. M	Tumour	Medium	Metabolism									
					Dye					Control				
					Q_{O_2}		$Q_G^{1/2}$		$Q_G^{1/2}$		Q_{O_2}		$Q_G^{1/2}$	
					1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.
Pot. ferricyanide	0.406	10^{-3}	JRS	Bic.	-11.5	—	8.8	—	23.0	—	-13.6	—	15.5	—
Bindschedler's green	0.224	10^{-4}	JRS	"	-18.8	—	17.3	—	—	—	-9.9	—	20.5	—
		10^{-4}	W/256	"	-23.2	-20.6	17.0	14.3	—	—	-11.1	—	22.5	—
		10^{-4}	W/256	Phos.	-2.2	—	—	—	—	—	-7.4	—	—	—
		10^{-4}	W/256	"	-20.2	—	—	—	—	—	-7.4	—	—	—
		3×10^{-5}	W/256	"	-10.3	—	—	—	—	—	-7.4	—	—	—
Tolylene blue	0.115	2.5×10^{-3}	W/256	"	-7.2	—	—	—	—	—	-7.4	—	—	—
		10^{-3}	JRS	Bic.	-12.8	—	+10.2	—	28.5	—	-8.4	—	22.0	—
		10^{-3}	JRS	"	-12.8	—	8.4	—	—	—	-13.7	—	13.5	—
		2×10^{-3}	JRS	Phos.	-15.7	—	—	—	—	—	-11.0	—	—	—
		5×10^{-3}	JRS	Bic. ($p_H 7.7$)	-28.0	—	26.2	—	—	—	-9.9	—	31.6	—
Thionine	0.062	6×10^{-5}	JRS	Phos. (dupl.)	-14.0	—	—	—	—	—	-8.1	—	—	—
		10^{-5}	JRS	"	-13.3	—	—	—	—	—	-9.0	—	—	—
		6×10^{-5}	JRS	Bic. ($p_H 7.7$)	-28.2	—	30.6	—	—	—	-12.2	—	31.6	—
		5×10^{-5}	W/256	Bic.	-26.8	—	14.6	—	—	—	-9.0	—	13.3	—
		5×10^{-5}	37/S	Phos.	-24.5	—	—	—	—	—	-8.9	—	—	—
Brilliant cresyl blue	0.045	5×10^{-4}	JRS	Bic.	-11.3	—	16.6	—	—	—	-5.2	—	25.7	—
		10^{-3}	JRS	"	-19.0	—	—	—	—	—	-11.0	—	19.8	—
		10^{-4}	JRS	"	—	—	—	—	38.2	20.4	—	—	—	—
		10^{-4}	JRS	"	—	—	—	—	38.2	38.1	—	—	—	—
		3×10^{-4}	W/256	"	-30.9	—	17.0	—	—	—	-11.2	—	21.3	—
		3×10^{-5}	W/256	"	-15.9	—	15.5	—	—	—	—	—	—	—
		4×10^{-5}	W/256	Phos.	-34.7	-7.8	—	—	—	—	—	—	—	—
		10^{-3}	W/256	"	-29.2	-17.0	—	—	—	—	—	—	—	—
		5×10^{-4}	"	"	-51.5	-16.5	—	—	—	—	—	—	—	—
		2×10^{-4}	"	"	-37.5	-23.0	—	—	—	—	—	—	—	—
		4×10^{-5}	"	"	-18.3	-15.7	—	—	—	—	-9.7	-8.6	—	—
		10^{-5}	"	"	-10.6	-8.1	—	—	—	—	—	—	—	—

[illegible]

toxic to kidney respiration. That the cells in which this increased respiration had occurred were still capable of growth was shown by transplantation into rats of carcinoma W/256 following 2 hours at 37.5° with 5×10^{-4} *M* brilliant cresyl blue in phosphate-glucose medium, during which period the average increases of respiration of the tumour slices were 128 %, 110 % and 103 % over the controls: there was a vigorous growth, similar to that of the control pieces, in all three experiments.

With these two dyes the R.Q. of the tumour was often considerably increased. Since in the absence of glucose an increased respiration is not observed with the dyes, it is probable that in tumour the oxidation of carbohydrate or lactate is increased; a result which would be in agreement with the value of the ratio (extra CO₂ : extra O₂) in presence of the dye, which is usually near to unity. This result is unlike that with pyocyanine or with methylene blue [Jares, 1935], where little increase of R.Q. usually occurs: the reason for this difference is not clear, though with methylene blue it might be explained by toxicity.

Brilliant cresyl blue and thionine therefore appear to be particularly advantageous for increasing the oxidation of tumours; however they do not restore the metabolism to normal, but appear rather to add a dye-catalysed carbohydrate oxidation to the ordinary respiration of the tumour, without effecting any qualitative alteration of the latter or of its influence on aerobic glycolysis.

Thionine and brilliant cresyl blue also increase markedly the oxidation of brain tissue in glucose media (Table V). Brain cortex in Ringer solution shows a small aerobic glycolysis: with methylene blue the respiration is increased 13 % [Barron, 1930], but with these two dyes increases of up to 190 and 100 % respectively have been observed.

Table V. *Brain cortex in glucose: percentage alteration of respiration.*

		Concentration (<i>M</i>)				
Substance added		2×10^{-4}	10^{-4}	5×10^{-5}	2×10^{-5}	10^{-5}
Thionine	Phos.	+ 81	—	+ 102	—	+ 55
	Bic.	—	—	+ 102	—	—
Brilliant cresyl blue	Phos.	+ 0	+ 95	—	+ 38	+ 26
	Bic.	—	+ 100	—	—	—
Phenosafranine	Phos.	- 24	- 30	- 2	—	+ 5
	Bic.	—	- 60	—	—	0
Neutral red	Bic.	—	- 10	—	—	—
Benzylviologen	Bic.	—	- 21	—	—	—
Phenazine methiodide	Phos.	—	+ 12	—	+ 10	—
	Bic.	—	+ 26	—	+ 9	—

Effect on aerobic glycolysis.

The most marked effect on aerobic glycolysis is the enormous increase caused by phenosafranine [Dickens, 1935] in both brain and tumour (Table IV). This will be dealt with in the following paper of this series.

Barron and Harrop [1928] showed that in erythrocytes the increased oxidation caused by methylene blue was accompanied by a fall in lactic acid; this however is explained by Warburg *et al.* [1930] as an oxidation of lactate to pyruvate brought about by the intermediate formation of methaemoglobin. A similar effect was not to be anticipated in ordinary body cells, and on the whole no fall of glycolysis was found to accompany increased oxidation caused by methylene

blue in tumour (Table IV); occasionally an increase was observed [cf. Gerard, 1931.] The less toxic dyes thionine and brilliant cresyl blue, despite very large increases caused in oxidation, often leave aerobic glycolysis quite unaffected. Their action is therefore not comparable with that of a normal respiration. Methyl Capri blue caused a slow continuous fall of aerobic glycolysis both with JRS and W/256.

It is clear that the action of dyes on aerobic glycolysis, like that on respiration, is complicated by irreversible toxic effects. For example, with brain tissue brilliant cresyl blue in higher concentration (10^{-4} M) causes at first an increased oxidation, which with this concentration of dye falls quickly, the aerobic glycolysis increasing simultaneously (Table VI). This effect is more marked with

Table VI. *Brain cortex in glucose: effect of brilliant cresyl blue.*

Animal	Conc. M	Medium	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Control		
						Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Rat	3×10^{-4}	Phos.	- 11.7					
	10^{-4}	"	- 23.0					
	3×10^{-5}	"	- 16.3					
	10^{-5}	"	- 14.9					
	10^{-4}	Bic.	- 21.6	5.8	26.2	12.4	0.8	20.2
		1st hr.	- 12.9	8.9	25.0	12.6	- 0.7	18.2
		2nd hr.	- 11.8	9.1	20.3	13.7	- 0.6	14.2
		3rd hr.	- 8.4	6.4	19.2	12.9	- 0.4	11.8
		4th hr.	- 21.3	9.0	27.4	- 10.4	+ 2.5	17.0
		1st hr.	- 17.7	1.0	24.1	- 9.1	3.6	13.1
Guinea-pig	10^{-4}	"	- 15.3	0.9	25.0	- 8.3	0.0	13.7

rat brain than with guinea-pig brain though KCl [Ashford and Dixon, 1935] affects both similarly (unpublished). Gerard [1931] reports increased glycolysis of brain caused by methylene blue.

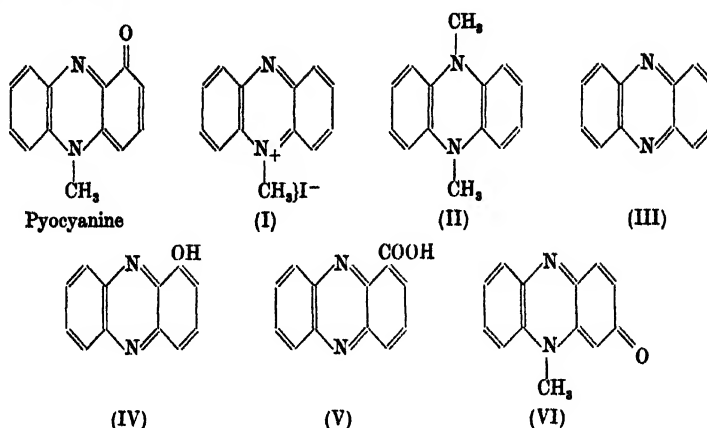
The strongly positive systems ferricyanide [Mendel, 1933], Bindschedler's green and toluylene blue (Table IV) cause a fall of aerobic glycolysis in tumour. This behaviour, though it is complicated by general toxic properties, would accord with the work of Lipmann [1933; 1934] who has demonstrated inhibition of fermentation in yeast and muscle extract by the more powerful oxidants.

Action of pyocyanine on glycolysis.

The action of pyocyanine [Friedheim, 1934] on aerobic glycolysis is of more interest since it appears to be possibly more nearly related to physiological processes. In Table IV the action of pyocyanine on JRS is shown and agrees with that found by Friedheim for other tumours. Respiration is increased with accompanying fall of aerobic glycolysis; the R.Q. however is not much raised as one would expect if the defective carbohydrate oxidation of the tumour were restored to equal that of normal tissue with similar glycolytic power [Dickens and Šimer, 1930]. Moreover pyocyanine, as will be shown, also decreases anaerobic glycolysis of tumours.

It appeared possible that, in addition to its effect in increasing oxidation, due to its action as a reversible oxidation-reduction system, pyocyanine might have an independent specific inhibitory effect on glycolysis. Accordingly the behaviour of some chemically closely related phenazine compounds, not known to behave as oxidation-reduction catalysts, has been studied. The formulae of

the substances tested, for which I am indebted to Prof. Clemo and Mr McIlwain, are shown below:



Phenazinemethiodide (I), which resembles pyocyanine in the presence of the *N*-methyl-group but differs from it in the absence of the α -oxy-group, has an action on tumour metabolism similar to that of pyocyanine (Table VII).

Table VII. *Action of phenazine compounds related to pyocyanine on tumour metabolism.*

Substance	Conc. <i>M</i>	Tumour	Medium	Metabolism			Control		
				Q_{O_2}	$Q_G^{1/2}$	$Q_G^{3/2}$	Q_{O_2}	$Q_G^{1/2}$	$Q_G^{3/2}$
Phenazine	4×10^{-4}	JRS	Bic.	-10.0	23.2	35.4	-9.8	23.1	36.3
Phenazine- α -carboxylic acid	10^{-3}	JRS	"	-11.5	23.8	—	-11.6	25.6	—
α -Hydroxyphenazine	10^{-4}	JRS	"	-8.9	20.0	38.4	-9.9	25.3	40.0
<i>NN'</i> -Dimethyldihydrophenazine	1.5×10^{-4}	JRS	"	-21.3	26.6	40.1	-10.3	25.1	46.1
		W/256	"	-14.3	14.4	—	-11.4	21.9	—
		Phos.	"	-14.0	—	—	-8.7	—	—
Phenazinemethiodide	3×10^{-4}	JRS	Bic.	{ -33.8 -27.0	{ 9.4 8.2	{ 18.0 —	-12.5	20.9	43.5
	3×10^{-5}	JRS	Phos.	-16.2	—	—	-8.7	—	—
Ordinary nitrogen	3×10^{-5}	JRS			1st hr. 29.0 2nd hr. 23.4 3rd hr. 22.6			1st hr. 39.5 2nd hr. 37.8 3rd hr. 41.2	
CrCl ₃ -purified nitrogen	3×10^{-5}	JRS			1st hr. 49.0 2nd hr. 33.2 3rd hr. 24.4				
	10^{-4}	W/256	Bic.	-27.8	12.0	—	-8.5	17.3	—
	3×10^{-5}	W/256	"	-28.4	18.8	—			
	10^{-4}	W/256	Phos.	-23.7	—	—	-8.4	—	—
	3×10^{-5}	W/256	"	-24.0	—	—			
Red oxidation product of <i>N</i> -methyldihydrophenazine	<i>ca.</i> 10^{-5}	JRS	Bic.	-6.6	20.9	28.0	-9.0	19.5	28.1

The respiration is greatly increased and with the higher concentrations the aerobic glycolysis falls at once whilst the anaerobic glycolysis is also slowly depressed. This substance is non-toxic to kidney respiration (Table I) in the concentration used and slightly increases brain oxidation with depression of aerobic glycolysis.

NN'-Dimethyldihydrophenazine (II) is sparingly soluble in salt solutions (approx. 10^{-4} *M*), nevertheless it increases tumour respiration and shows a

tendency to depress aerobic glycolysis comparable with that of phenazinemethiodide in similar concentration.

These compounds are both free from substituents attached to the C atoms of the phenazine ring. It might therefore be concluded that the oxy-group is not necessary for the pyocyanine type of action. This, however, is not certain, since the compounds may undergo oxidation within the tissue. In the case of phenazinemethiodide this is actually observed: when the tissue slices are shaken in oxygen with phenazinemethiodide, itself very pale yellowish green, they become stained a brick-red colour, which is discharged on transferring the tissue to nitrogen and returns again on re-admitting air. Kehrman [1913] obtained a red oxidation product, not isolated in the pure form, probably *N*-methylaposafranone of structure VI, from alkaline solutions of methylphenazonium salts; the same worker [1924] also obtained a similar product from the methosulphate of 2-hydroxyphenazine. Hantzsch [1916] mentions the formation of phenazine and a red substance by air oxidation of *N*-methyl-dihydrophenazine. An attempt was made to test the hypothesis that the red compound formed in the tissue is responsible for the catalytic action and that it is of similar type to these oxidation products. Unfortunately the solubility in salt solution of the red compound VI, prepared by the method of Hantzsch [1916], was found to be exceedingly small, and the result is therefore inconclusive (Table VII).

NN'-Dimethyldihydrophenazine appears structurally unsuited for a respiratory catalyst, but it is readily oxidised [cf. Clemon and McIlwain, 1935] and probably undergoes oxidation before it exerts its effect. The activity of these two compounds and their chemical relationship to pyocyanine indicate that in these substances the presence of the *N*-methylphenazine structure is associated with the specific inhibitory effect on glycolysis as well as with increased oxidation. On the other hand, when the *N*-methyl-group is absent, as in α -carboxyphenazine (V) and the sparingly soluble unsubstituted phenazine (III), these properties are lost. Although α -hydroxyphenazine (IV) shows a measurable oxidation-reduction potential ($E^\circ_h = 0.17$ volt [Michaelis 1931]) its action on tumour metabolism is not marked (Table VI), and is of a very different order from that of pyocyanine or of the two *N*-substituted phenazines tested. It is of interest in this connection that the introduction of amino-groups, as in phenosafranine (3:6-diamino-10-phenylphenazonium chloride) entirely reverses the activity of the substituted phenazine compounds, so that instead of an inhibition of glycolysis an increase is observed, the Pasteur mechanism being put out of action by phenosafranine. This action will be described in the next paper of this series.

Action on anaerobic glycolysis.

As with aerobic glycolysis, the strongly positive systems ferricyanide, 2:6-dichlorophenolindophenol and toluylene blue caused a decrease in anaerobic glycolysis. Methylene blue and phenosafranine in these experiments had little or no effect. The action of brilliant cresyl blue is of interest, since with brain a marked increase of anaerobic glycolysis above the normal level occurred (Table VI). With Jensen sarcoma, on the other hand, there was no effect for about 60 min., after which a fall in anaerobic glycolysis occurred, whether ordinary commercial nitrogen or nitrogen in presence of chromous chloride as oxygen absorbent was used as the gas phase.

The action of pyocyanine in purified nitrogen showed a resemblance to that of brilliant cresyl blue; in presence of chromous chloride the anaerobic glycolysis remained nearly constant for about 60 min. and then fell rapidly. When ordinary commercial nitrogen, containing 0.3% O_2 , was used instead, the glycolysis was

inhibited from the beginning of the experiment. Inhibition of glycolysis only occurred in N_2 when the concentration of pyocyanine was sufficient for part of it to remain oxidised throughout; in experiments where complete decoloration of the pyocyanine occurred there was little or no inhibition of glycolysis (Table IV). From these facts it seems probable that inhibition of glycolysis is brought about by the oxidised form of pyocyanine, and that it is necessary for a limiting concentration of this form to be present within the tissue if inhibition of glycolysis is to occur. The presence of oxygen, even in traces, is sufficient to ensure this; if oxygen is completely absent, as in the $CrCl_3$ experiments, for a period of about 60 min. the reducing systems of the tissue are able to deal with the pyocyanine which penetrates the cell; after this period they do not appear to be able to react quickly enough to keep the pyocyanine reduced. Consequently the oxidised pyocyanine predominates and the glycolysis is inhibited.

In comparison with pyocyanine the action on anaerobic glycolysis of other phenazines is of interest. Phenosafranine has little effect, phenazine none and α -hydroxyphenazine may cause a slight decrease of about 20% in $3 \times 10^{-4} M$ concentration. Phenazinemethiodide causes a slow fall, while dimethyldihydrophenazine is too sparingly soluble to give a sufficiently concentrated solution to show any effect.

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CLV. STUDIES ON THE MODE OF ACTION OF VITAMIN K.

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SCHØNHEYDER [1935; 1936, 1, 2] has shown that the most probable explanation of the low clotting power of blood from K-avitaminous chicks is a reduced content of prothrombin in the plasma.

The present brief investigation lends definite support to this view, because it has been possible to precipitate, by the acetone method of Howell [1911] and the acetic acid method of Mellanby [1930], the prothrombin from the plasma of normal chicks, whilst a corresponding precipitate from the plasma of K-avitaminous chicks could be shown to be inactive as prothrombin.

The fact that a fat-soluble vitamin is involved in the maintenance of the necessary amount of prothrombin in the blood of chicks would be explained if prothrombin contained a lipid component. Prothrombin is, as commonly known, water-soluble and follows the globulin fraction in many precipitation reactions.

When the prothrombin precipitate was washed several times with acetone and ether, whereby lipoids, including carotene pigments, are removed, the washed precipitate was still found to be active, and the lipoids which passed into solution did not show any activity as prothrombin. These facts do not, of course, exclude the possibility that vitamin K, or a derivative thereof, might be present in the prothrombin as a prosthetic group which is held in firm combination with the rest of the molecule.

The washed prothrombin precipitate was, by a single test, found to contain vitamin K.

Further investigation with more elaborate methods of concentrating the prothrombin must decide whether vitamin K is an essential constituent of the proenzyme.

A direct test of vitamin K for prothrombin activity was carried out and gave a negative result.

EXPERIMENTAL.

Precipitation of prothrombin from the plasma of normal and K-avitaminous chicks.

1. *Acetone method* [Howell, 1911].

(a) *Normal.* 3 ml. plasma were mixed with an equal volume of acetone at 0° and immediately filtered on a suction filter. After washing with ether the precipitate was dried on the filter-paper in a vacuum desiccator and dissolved in 3 ml. Ringer's solution; undissolved particles were removed by centrifuging.

(b) *K-avitaminous.* 3 ml. plasma were treated as above and the precipitate was dissolved in 3 ml. Ringer's solution.

2. *Modified acetone method.*

(a) *Normal.* 4 ml. plasma were mixed with 4 ml. acetone at 0°, centrifuged and the precipitate washed with acetone and ether, all these operations being

performed at 0°; after drying in a vacuum desiccator, the precipitate was dissolved in 4 ml. Ringer's solution.

3. *Acetic acid method* [Mellanby, 1930].

(a) *Normal*. 30 ml. plasma were diluted with 300 ml. water at 0° and brought to p_H 5.3 by means of 1 % acetic acid. After 1 hour the precipitate was separated by centrifuging, washed with acetone and ether and dried in a vacuum desiccator. All operations up to the centrifuging were performed in paraffin-coated vessels at 0°; weight of the precipitate, 239 mg. 7.9 mg. were dissolved in 1 ml. Ringer's solution.

(b) *K-avitaminous*. 5 ml. plasma were treated in the same way as (3a); weight of the precipitate, 30 mg. The whole was dissolved in 5 ml. Ringer's solution.

Preparation of an emulsion of the lipoids.

4. 9 ml. plasma were allowed to stand with 10 ml. alcohol (96 %) for 24 hours, centrifuged and the precipitate washed twice with alcohol and three times with ether. The combined liquids were taken to dryness *in vacuo* and extracted with ether. The evaporated ether extract was dissolved in acetone and allowed to flow in small droplets into 9 ml. water with constant shaking; after evaporation of the acetone *in vacuo* the solution was made up to 9 ml.

Preparation of an emulsion of the lipoids from the prothrombin preparation (2 a).

5. The combined acetone and ether washings were dried *in vacuo*, dissolved in acetone and emulsified as above. The volume of the emulsion was made up to 2.5 ml. after evaporation of the acetone.

Emulsion of a concentrate of vitamin K.

6. 8.8 mg. of a concentrate of vitamin K from alfalfa, containing 650,000 units per g., were dissolved in light petroleum (10 ml.). 1 ml. of this solution was taken to dryness *in vacuo*, dissolved in acetone and mixed with 1 ml. of distilled water. After evaporation of the acetone *in vacuo* the volume was adjusted to 1 ml. 1 ml. of the emulsion contains 570 units.

Dried plasma.

7. Plasma from 2 normal hens was taken to dryness in a vacuum (CaCl_2) desiccator, spread in a thin layer on porcelain discs, and made into tablets. This plasma was practically colourless.

Prothrombin precipitate for vitamin K test.

8. 940 mg. precipitate were prepared from 99 ml. normal plasma (intense yellow) from 4 normal hens, the acetic acid method described under (3a) being used. The precipitate was tested for prothrombin and made into tablets.

Lipoids from normal plasma for vitamin K test.

9. 60 ml. normal plasma (intense yellow) were stirred with 120 ml. 96 % ethyl alcohol, placed in ice for 30 min., centrifuged and the precipitate washed with alcohol and ether until the ether remained colourless. The liquids were evaporated and the residue extracted with ether. The combined extract weighed 748 mg.

Tests for prothrombin activity. As test substrate plasma from K-avitaminous chicks with an *S* value above 2000 was used [cf. Schönheyder, 1936, 2]. This plasma was diluted with Ringer's solution—equal parts—and a certain amount (measured by means of a dropping pipette giving 20 droplets per ml.) of the

preparation to be tested as well as a thrombokinase solution were added. Thrombokinase from hen's muscle is available in the form of "Thrombisol" a watery solution prepared by the firm "Leo" Copenhagen. Corresponding experiments without thrombokinase served as controls for thrombin activity of the preparations. The apparatus used for determining the clotting time was that of Fischer [1930; see Schönheyder, 1936, 2]. The "thrombisol" must be so concentrated that it gives a suitable clotting time, *e.g.* 5–7 min., with the plasma from K-avitaminous liver when no prothrombin is added (*cf.* the experiments in which Ringer's solution was used instead of thrombokinase). If necessary, the thrombisol must be diluted with Ringer's solution before use. The activity of the prothrombin preparations is demonstrated by the difference in clotting time between the experiments with Ringer's solution and those with "prothrombin". The clotting times in the experiments with lipid emulsions are to be compared with the corresponding experiments with distilled water instead of emulsion.

The results appear in Table I, the plasma and thrombokinase being the same in each experiment.

Table I.

Exp. no.	Preparation	Droplets	Diluted "K-avitaminous" plasma droplets	Thrombo-kinase droplets	Clotting time (sec.)
1	Ringer's solution	1	5	1	285, 300, 315, 300
	Prothrombin, normal 1 <i>a</i>	1	5	1	130, 130
	Normal hen's plasma	1	5	1	85, 85, 85, 85
	Prothrombin, normal 1 <i>a</i>	1	5	0	>600, >600
	Prothrombin, sick 1 <i>b</i>	1	5	1	315, 315
	Prothrombin, sick 1 <i>b</i>	1	5	0	>600, >600
2	Ringer's solution	1	5	1	390, 390, 405, 435 450, 435, 450, 465
	Prothrombin, normal 3 <i>a</i>	1	5	1	135, 135
	Prothrombin, normal 3 <i>a</i>	1	5	0	>900, >900
	Prothrombin, sick 3 <i>b</i>	1	5	1	375, 390, 375, 390
	Prothrombin, sick 3 <i>b</i>	1	5	0	>720, >720
3	Lipoids of normal plasma 4	3	3	1	210, 210
	Distilled water	3	3	1	195, 210, 210, 210
4	Ringer's solution	1	5	1	195, 225, 210, 210 195, 180
	Prothrombin, normal 2 <i>a</i>	1	5	1	90, 90, 90
	Prothrombin, normal 2 <i>a</i>	1	5	0	>1800, >1800
	Distilled water	1	5	1	165, 165
	Lipoids from prothrombin 3	1	5	1	170, 170, 180, 180
	Equal parts of 2 <i>a</i> and 5	1	5	1	105, 105, 105, 105 105, 90
	Equal parts of 2 <i>a</i> and 5	1	5	0	>1800, >1800
5	Distilled water	2	4	1	240, 285
	Vitamin K concentrate 6	2	4	1	270, 300
6	Distilled water	1	4	1	345, 345
	Vitamin K concentrate 6	1	4	1	345, 345

Tests for vitamin K. The method of Schönheyder [1936, 2] was used.

1. 3×1 g. dried plasma were given to a chick weighing 233 g. The *S* value was found to be 160, corresponding to a vitamin K content of 46 units per g. dry matter.

2. 3×310 mg. prothrombin preparation were given to a chick weighing 192 g. *S* = 180 corresponding to 142 units per g.

3. 3×222 mg. lipid extract were given to a chick weighing 233 g. *S* = 143, corresponding to 323 units per g. lipid.

DISCUSSION.

Exp. 1 shows that the prothrombin precipitate (1a) from a normal chick (Howell's acetone method) accelerates the clotting time from about 300 sec. to 130 sec., whilst the corresponding precipitate (1b) from a K-avitaminous chick has no influence. The normal plasma itself has a somewhat stronger action than the prothrombin preparation (1a) (acceleration to 85 sec.). The effect of the prothrombin preparation (1a) is not due to thrombin, since the preparation without kinase does not clot the plasma within the time of observation (600 sec.).

Exp. 2 shows corresponding results for prothrombin precipitated by acetic acid according to Mellanby (3a and 3b).

Exp. 3 shows that the lipoids of normal plasma do not accelerate clotting.

Exp. 4 shows that the prothrombin precipitated from normal plasma by acetone (2a) in a modified way which secures a better removal of lipoids than the original Howell method, also accelerates the clotting, and that the lipoids (5) which are removed from the precipitate during washing with acetone and ether do not have any accelerating effect. A mixture of the prothrombin preparation (2a) and the lipoids (5) is no better than the prothrombin itself.

Exps. 5 and 6 show that vitamin K does not act as prothrombin *in vitro*. The number of K-units which have been added in Exp. 5 is 57 in 7 droplets of liquid = 0.35 ml., corresponding to 163 units per ml. This is a very large amount as compared with the content of normal plasma. The pale normal plasma (7) contained 46 units of vitamin K per g. of dry matter, which is about 3 units per ml.

The lipid fraction and the prothrombin fraction of intensely yellow plasmata were found to contain 323 and 142 units per g. respectively. It is thus possible that vitamin K circulates in the plasma in two different forms, one of which is easily extractable with acetone and ether whilst the other is more firmly combined with the proteins. Further investigation is, however, required to settle this question and to ascertain whether vitamin K is also a constituent of more concentrated prothrombin preparations.

Certain authors [Nolf, 1905; 1908; Gratia, 1914] have suggested that prothrombin is formed in the liver. It might therefore be expected that vitamin K would act through the liver of the chick and be stored there. It is, in this connection, interesting to note that Dam and Schønheyder [1936] found very little vitamin K in the liver of a normal chick—less than 11 units per g. dry matter, a figure which is much smaller than that found in the plasma.

SUMMARY.

1. It is possible to precipitate prothrombin from the plasma of normal chicks by means of the acetone method of Howell and the acetic acid method of Mellanby, whilst the corresponding precipitates from the plasma of K-avitaminous chicks are inactive as prothrombin.

2. The prothrombin precipitate from normal chicken plasma is still active after removal of lipoids by acetone and ether, and the lipid obtained in this way has no prothrombin activity. This also holds good for the lipid which can be extracted from the plasma itself by alcohol and ether.

3. A concentrate of vitamin K was found not to accelerate clotting *in vitro*, when tested against plasma + thrombokinasase.

4. The prothrombin preparation precipitated by acetic acid (Mellanby) and washed with acetone and ether was found to contain vitamin K. The interpretation of this result, however, requires further investigation.

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CLVI. STUDIES ON AMINO-ACID DEHYDROGENASE.

I. PROLINE DEHYDROGENASE.

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It has been shown by many workers, especially by Knoop [1925] and Krebs [1933], that amino-acids are oxidised by tissues *in vitro*. It has later been reported by Bernheim and Bernheim [1934; 1935] and Krebs [1935] that a cell-free extract of tissues can also oxidise aerobically many amino-acids, and the former authors claim to have obtained an enzyme from tissue extracts which can decolorise methylene blue more rapidly in presence of proline, phenylalanine, valine, leucine, serine *etc.*

The present work is a study of the mechanism of oxidative deamination of amino-acids by cell-free dehydrogenases. It has been shown that dehydrogenases such as alcohol dehydrogenase, glucose dehydrogenase, hexosemonophosphate dehydrogenase *etc.* require the presence of a low-molecular co-enzyme; that is to say the active enzyme (holo-dehydrogenase) consists of a high-molecular group (apo-dehydrogenase) and a low-molecular prosthetic group (co-dehydrogenase), the components of the holo-dehydrogenase being in dissociation equilibrium. Up to the present time two co-enzymes (co-dehydrogenases) are known, namely co-enzyme (co-dehydrogenase I) and the co-ferment of Warburg and Christian [1935] (co-dehydrogenase II).² These co-enzymes act by taking up activated hydrogen from the substrate, thereby forming the reduced co-enzymes which are not autooxidisable but are reoxidised by flavin-enzyme. The leucoflavin-enzyme itself can act directly with molecular oxygen or with a reducible dye like methylene blue. It seemed to be of interest to investigate whether amino-acid dehydrogenase has anything to do with co-enzyme-flavin-enzyme catalysis. The fact that amino-acid oxidation by cell-free extracts was not inhibited by KCN made it possible that it might be such a metal-free system in which co-enzyme-flavin-enzyme catalyses the reaction.

In the present work the problem has been studied with reference to proline. None of the known activators like co-enzyme (Euler), co-dehydrogenase II [Warburg and Christian, 1935; Euler and Adler, 1935, 3; 1936], flavin-enzyme [Warburg and Christian, 1932, 1, 2], glutathione, ascorbic acid, adenylypyrophosphate can activate proline dehydrogenase except glutathione in higher concentrations. (This may be due to some inhibiting metal which cannot be bound by lower concentrations of glutathione.) The facts that the dehydrogenase is not catalysed by flavin-enzyme and that anaerobic reduction of dyes is relatively much slower than the oxidation by molecular oxygen seem to indicate that flavin-enzyme is not a necessary component of the system.

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² For the general nomenclature of the components of dehydrogenases see Euler and Adler [1936].

It has been found that the original enzyme solution heated at 80 to 90° for 5 min. at p_H 8.0 can activate proline dehydrogenase. This activator is destroyed when heated at p_H 3.0 and p_H 12.0 for 5 min. at 90° and is also inactivated by ultraviolet irradiation. The chemical nature and the specificity of this activator have not yet been sufficiently studied but further investigations are in progress.

EXPERIMENTAL.

Extraction of the enzyme.

The enzyme was extracted according to Krebs [1935] from acetone-dried pig kidney, 1 g. being extracted with 40 ml. distilled water for 10 min. and centrifuged. The centrifugate could oxidise proline, alanine, phenylalanine and leucine. 2 ml. of this extract were used for every experiment.

Method of experiment.

The oxidation experiments were carried out in Warburg-Barcroft apparatus at 30°. Phosphate buffer (p_H 7.8, $M/20$) was always used with the enzyme solution. Substrate was always added from the side-limb after equilibration of the temperature of the vessels and 0.4 ml. of 8% NaOH was used for absorbing the CO_2 formed.

Purification of the enzyme.

(a) *Adsorption with aluminium oxide C γ* (Willstätter). 30 ml. of the enzyme solution prepared from acetone-dried pig kidney were stirred with C γ suspensions for 15 min. at p_H 6.0 and centrifuged. The centrifugate still contained most of the proline dehydrogenase.

(b) *Adsorption with kaolin*. 40 ml. of the enzyme solution were agitated with 2 g. kaolin for 15 min. at p_H 6.0 and centrifuged. The centrifugate had lost a considerable portion of the proline dehydrogenase.

The enzyme was eluted from the kaolin by phosphate buffer (p_H 7.8, $M/20$). To the kaolin adsorbate 9 ml. of phosphate buffer and 1 ml. of 1% Na_2CO_3 were added, stirred for 30 min. and centrifuged. The centrifugate was found to oxidise proline. The enzyme solution eluted from kaolin in this way was used throughout this work, 1 ml. being employed for all experiments described below.

Nature of the enzyme.

The light brown clear enzyme solution could hardly oxidise *D*-alanine, whilst with *DL*-proline oxidation was extensive. The velocity of reaction was very great at the beginning and then relatively slow. The enzyme was unstable at p_H 8.0 at room temperature but more stable in the cold.

Test for activators.

In order to see whether co-enzyme could act as an activator in the oxidation of proline by the enzyme 1 ml. of the enzyme solution freshly prepared from pig kidney was tested in yeast fermentation according to the well-known method of Myrbäck and Euler [1929], but no appreciable evolution of CO_2 was found even after 1 hour.

It has been shown that in fresh tissue extracts an inhibiting substance is present which prevents the reaction with co-enzyme and that this inhibitor can be destroyed by heating [Meyerhof, 1918; Euler, Adler and Dahlgren, 1935]. The enzyme solution was therefore heated for 5 min. at 80° and tested as before on fermentation. In this case also however no appreciable amount of CO_2 was formed. Various kidney preparations were tested in this way and only in one preparation was there very slight evolution of CO_2 (0.75 CO_2 /ml.).

1. *Co-enzyme*. Co-enzyme has been found by many workers, especially by Andersson [1934], to activate many dehydrogenases. This wide range of co-

zymase as an activator suggested its possible rôle in the oxidation of amino-acids. Co-zymase, prepared from yeast according to Euler, Albers and Schlenk [1935], was therefore added to the enzyme but no increased oxygen uptake was found with proline, suggesting that co-zymase plays no part in the activation of proline dehydrogenase. Various preparations of co-zymase have been tried.

Table I.

No.	Enzyme solution ml.	Co-zymase ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	37.0	92.4	147.8	195.4
2	1.0	0.3	1.0	0.1	40.1	92.8	137.4	175.5
3	1.0	0.3	1.0	0.1	41.2	89.7	131.0	172.2
4	1.0	—	1.0	0.1	50.2	105.6	145.2	172.2
5	1.0	0.3	1.0	0.1	55.8	111.5	150.3	179.4
6	1.0	—	1.0	—	—	—	—	—

2. *Co-dehydrogenase II*. (a) Warburg's co-ferment from horse red blood corpuscles, prepared according to Warburg and Christian [1933] acts as co-enzyme in the oxidation of hexosemonophosphate. It has been shown [Das, 1936] that it can also act as co-enzyme to glucose dehydrogenase. It fails however to activate the oxidation of proline (Table II).

(b) *Co-dehydrogenase II from yeast*. It was found by Euler and Adler [1935, 3; 1936] that impure co-zymase from yeast contained an activator for the oxidation of hexosemonophosphate which was named co-dehydrogenase II and was identical with Warburg and Christian's co-ferment from blood-cells. This also could activate glycolase dehydrogenase [Das, 1936] but failed to activate the oxidation of proline (Table II).

Table II.

No.	Enzyme solution ml.	Co-dehydrogenase II (ml.)	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	37.0	92.4	147.8	195.4
2	1.0	0.3*	1.0	0.1	32.1	81.6	128.2	171.9
3	1.0	0.3†	1.0	0.1	34.0	86.0	136.0	180.0

* Euler and Adler [1935, 3; 1936].

† Warburg and Christian [1933].

3. *Flavin-enzyme*. Warburg and Christian [1932, 1, 2] isolated a yellow pigment from bottom yeast which is a protein combined with a yellow prosthetic group and is capable of reversible oxidation and reduction. It has been shown that the enzymic oxidation of alcohol and glucose either by molecular oxygen, methylene blue or lactoflavin is catalysed by flavin-enzyme [Euler and Adler, 1934; 1935, 1]. It was also shown by Euler, Adler and Hellström [1935] that reduced co-enzyme was reoxidised by flavin-enzyme, but the latter failed to activate proline dehydrogenase (Table III).

Table III.

No.	Enzyme solution ml.	Flavin-enzyme ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	13.2	42.2	84.5	121.4
2	1.0	0.5	1.0	0.1	24.2	50.9	89.7	126.0
3	1.0	—	1.0	0.1	50.2	105.6	145.2	174.2
4	1.0	0.5	1.0	0.1	59.0	108.9	146.3	174.3

4. *Adenylpyrophosphate (A.T.P.)*. Adenylpyrophosphate is involved in the activation of several reactions such as muscle glycolysis [Lohmann, 1931] and as a

phosphate donator to carbohydrates [Euler and Adler, 1935, 2; Parnas *et al.*, 1935; Lutwak-Mann and Mann, 1935]. A preparation of adenylypyrophosphate from rabbit muscle was incapable of activating the oxidation of proline by the dehydrogenase (Table IV).

Table IV.

No.	Enzyme solution ml.	A.T.P. ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	29.0	79.2	116.2	147.8
2	1.0	0.3	1.0	0.1	33.9	65.5	89.7	109.1

5. *Ascorbic acid*. The reversible oxidation-reduction of ascorbic acid suggests the possibility of its important rôle in oxidation-reduction systems of the body. It has been shown to be capable of activating intracellular proteases of the type of cathepsin, which by washing with acetone and alcohol have been freed from their natural activators [Karrer and Zehender, 1933]. Ascorbic acid however is also unable to activate the oxidation of proline by the dehydrogenase (Table V).

Table V.

No.	Enzyme solution ml.	Ascorbic acid ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					10	20	40	60
1	1.0	—	1.0	0.1	11.7	29.14	72.9	102.0
2	1.0	0.5 (1 mg.)	1.0	0.1	19.4	38.8	65.5	82.4
3	1.0	—	1.0	0.1	32.1	51.2	107.8	145.7
4	1.0	0.5 (2 mg.)	1.0	0.1	30.3	49.5	82.5	107.3

6. *Glutathione*. Since the discovery of glutathione by Hopkins [1929] many speculations have been made as to its rôle in metabolism. It was shown by Mann [1932] that glutathione is reduced by glucose dehydrogenase and later by Meldrum and Tarr [1935] that it is also reduced by the Warburg and Christian hexosemonophosphate oxidising system. It has also been studied as an activator in various reactions, and it therefore seemed to be of importance to study its rôle in the oxidation of amino-acids by the dehydrogenase. Various concentrations were used and it was found that a concentration of $M/300$ activated the system whilst a smaller concentration ($M/1500$) had no effect (Table VI).

Table VI.

No.	Enzyme solution ml.	Glutathione ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	21.1	50.2	81.8	108.2
2	1.0	0.1 $M/300$	1.0	0.1	52.6	97.8	137.9	168.0
3	1.0	0.1 $M/1500$	1.0	0.1	28.0	49.8	77.8	99.6

Action of dyestuffs on the enzyme.

The anaerobic reduction of methylene blue by the enzyme was very slow in absence of proline and was only slightly more rapid in presence of the substrate [Bernheim and Bernheim, 1934; 1935]. The effect of the dye on the aerobic oxidation of proline was then studied and was found to be slightly inhibitory in a concentration of $M/60,000$. A dye with more positive redox potential, 2,6-dichlorophenolindophenol, was also studied but was found to have no effect in a concentration of $M/60,000$ (Table VII).

Table VII.

No.	Enzyme solution ml.	Dyestuffs ml. <i>M</i> /60,000	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O ₂ uptake after min.			
					10	20	40	60
1	1.0	—	1.0	0.1	57.6	118.0	222.7	288.2
2	1.0	0.3*	1.0	0.1	47.6	102.8	205.6	268.2
3	1.0	0.3†	1.0	0.1	36.4	80.0	167.3	228.0

* 2:6-Dichlorophenolindophenol.

† Methylene blue.

Action of inhibitors on the enzyme.

1. *KCN*. *KCN* in the concentration of *M*/100 had a slight activating effect on the dehydrogenase. This might be due to some metal which was present in the original enzyme solution. The original enzyme solution was therefore treated with *KCN* and kept at room temperature (20°) for 1 hour, but the same slight activating effect was found as when *KCN* was added along with the substrate after equilibration of temperature of the vessels (Fig. 1).

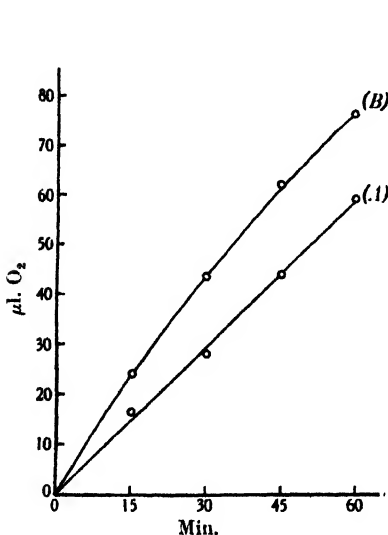


Fig. 1.

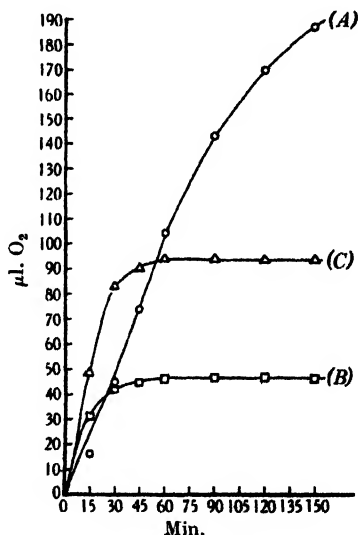


Fig. 2.

Fig. 1. Oxygen uptake in μ l. (A) Enzyme and proline; (B) enzyme, proline and *M*/100 *KCN*.

Fig. 2. Oxygen uptake in μ l. (A) Enzyme and proline; (B) enzyme, proline and *CuSO*₄ (*M*/75); (C) enzyme, proline, *CuSO*₄ (*M*/75) + *KCN* (*M*/100).

2. *CuSO*₄ and *CuSO*₄ + *KCN*. Both *CuSO*₄ and *CuSO*₄ + *KCN* when added to the enzyme had activating effects for a short time, followed by complete inactivation of the enzyme. The initial activation might be due to the formation of a copper compound of the substrate which had a more labile hydrogen, whilst copper itself acted as a poison to the enzyme. The action of *CuSO*₄ + *KCN* was the same as that of *CuSO*₄ itself. The slight increase of initial oxygen uptake with the "Cu-complex" might be due to the slight activating action of *KCN* (Fig. 2).

Action of H_2O_2 on the enzyme.

It was shown by Keilin and Hartree [1936] that the H_2O_2 formed during the oxidation of xanthine by xanthine oxidase destroys the enzyme. It has also been shown that H_2O_2 is formed during oxidation of amino-acids by amino-acid dehydrogenase. To see whether the decrease in velocity of the oxidation of proline after a short time is due to the destruction of the enzyme by H_2O_2 formed during the course of reaction, a solution of H_2O_2 was added with the substrate after equilibration of temperature to make 1% concentration; no inhibition was observed however even after 90 min. (Table VIII).

Table VIII.

No.	Enzyme solution ml.	H_2O_2 1%	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O_2 uptake after min.			
					15	30	60	90
1	1.0	—	1.0	0.1	10.6	34.3	76.6	110.9
2	1.0	0.1	1.0	0.1	10.0	31.4	68.1	104.8

Heated enzyme solution as activator.

The possibility was next considered that the activator might have been bound up with some protein carrier which could be split off by heating. The enzyme solution was therefore heated at 80° for 5 min. at p_H 8.0. The heated enzyme solution, when added to the original enzyme, produced a great increase in the oxidation of proline which was not due to the presence of residual enzyme or any substrate.

The ash of an enzyme preparation had no effect on the enzyme, showing that the activation by the heated solution was not due to the presence of any metal (Table IX).

Table IX.

No.	Enzyme solution ml.	Heated enzyme solution ml.	Ash of the enzyme solution	Buffer ml.	<i>M</i> proline ml.	μ l. O_2 uptake after min.			
						15	30	45	60
1	1.0	—	—	1.0	0.1	29.0	79.2	116.2	147.8
2	1.0	0.5	—	1.0	0.1	78.7	163.2	224.4	268.1
3	1.0	—	0.5 (5 mg.)	1.0	0.1	37.6	82.7	127.9	162.0
4	—	0.5	—	1.0	0.1	—	—	—	—
5	1.0	0.5	—	1.0	—	—	—	—	—

That the activation was not due to co-enzyme or co-dehydrogenase II was also proved by testing the heated enzyme preparation on yeast fermentation as well as on the hexosemonophosphate oxidising system; furthermore, KCN did not inhibit the activation, showing that neither catalase nor peroxidase is responsible (Table X).

Table X.

No.	Enzyme solution ml.	Heated enzyme solution ml.	KCN <i>M</i> /110	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O_2 uptake after min.			
						15	30	45	60
1	1.0	—	—	1.0	0.1	10.6	34.3	76.6	110.9
2	1.0	—	0.3	1.0	0.1	32.1	67.0	107.8	145.7
3	1.0	0.5	—	1.0	0.1	50.1	100.1	170.5	228.1
4	1.0	0.5	0.3	1.0	0.1	65.4	126.0	184.2	242.4

Negative nitroprusside tests or tests of an intensity lower than that corresponding with effective concentrations of glutathione were given by the heated solution; it is clear therefore that the natural activator cannot be glutathione.

Nature of the activator.

1. *Stability of the activator.* In order to study the stability of the activator the original enzyme preparation was heated at 90° for 5 min. at p_H 3, 5, 6, 7, 8, 9, 10, 11, 12. It was found that the activity was partially destroyed at p_H 11.0 and completely destroyed at p_H 3.0 and 12.0, whilst at other p_H values the activity was fairly constant.

When the enzyme was heated at $p_H < 8.0$ a heavy precipitate was formed; the clear colourless filtrate contained the activator.

2. *Destruction by ultraviolet rays.* 3 ml. of the heated enzyme solution were exposed to ultraviolet rays in an open small crystallising dish for 15 min. The activator was found to be completely destroyed.

3. *Dialysis.* The activator was found to be very slowly dialysable. After 4 hours' dialysis in a cellophane tube against running water about 10 % and after 20 hours' dialysis about 80 % of the activity was found to be lost.

Anaerobic reduction of dyestuffs.

Though both methylene blue and 2:6-dichlorophenolindophenol were reduced very slowly in presence of the enzyme and proline, the reduction was comparatively rapid in presence of the heated enzyme, showing that the heated enzyme could also act as an activator in the anaerobic reduction of dyes (Table XI).

Table XI.

Enzyme solution heated at 90° for 5 min. at p_H 8.0 was used as the activator solution. Substrate: *M* proline. *M*/20 phosphate buffer at p_H 7.8 and 2:6-dichlorophenolindophenol 1 : 5000. Total volume of the fluid 3 ml. Thunberg technique was followed.

No.	Enzyme solution ml.	Activator solution ml.	Phosphate buffer ml.	<i>M</i> proline ml.	Dye 1/5000 ml.	Time of decoloration in min.
1	1.0	0.25	0.4	0.1	0.5	58
2	1.0	—	0.4	0.1	0.5	32
3	1.0	0.25	0.4	—	0.5	> 180

SUMMARY.

1. A purified enzyme has been studied which can oxidise *DL*-proline more than *D*-alanine, *DL*-phenylalanine and leucine.

2. Several known activators such as co-enzyme (co-dehydrogenase I), Warburg co-ferment (co-dehydrogenase II), flavin-enzyme, glutathione, adenylypyrophosphate and ascorbic acid failed to activate the proline dehydrogenase.

3. When the original enzyme solution was heated at 80° at p_H 8.0 for 5 min. and added to the original enzyme an activation of the enzymic oxidation of proline was found.

The author is grateful to Prof. Hans von Euler and Dr E. Adler for advice and criticism.

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CLVII. BRAIN PHOSPHATASE.

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(Received March 9th, 1936.)

KAY [1928] AND KING [1931] have shown that the brain of animals contains a phosphatase which hydrolyses glycerophosphate. Recently Edlbacher *et al.* [1934] have investigated the enzymes of brain and have come to the conclusion that the brain substance causes a slight cleavage of nucleic acid, magnesium hexosephosphate and sodium glycerophosphate. Except for the above findings very little is known about the phosphatases of the brain. Brain phosphatase is of interest since it is present in an organ rich in phosphatides. In the present work the nature and action of brain phosphatase and its identity with the phosphatases present in other organs have been investigated.

EXPERIMENTAL.

Preparation of material. Sheep's brain was used. The whole brain of each animal (weighing about 80–90 g.) was obtained just after the animal was killed, washed with distilled water and trimmed free from the outer membrane with capillaries containing blood. The tissue was ground in a mortar, the pulp stirred with 200 ml. of acetone for 15 min. and then filtered on a Büchner funnel. The process was then repeated, followed by washing with 200 ml. of ether. The dry powder was spread in a thin layer on filter-paper and left overnight at room temperature. The next day it was ground in a mortar to a fine powder and stored in a vacuum desiccator. The white, fat-free brain powder thus obtained amounted to about 10–12 g. This powder keeps its activity for several months.

Enzyme. The powder was treated with ten times the volume of distilled water containing toluene and allowed to remain at room temperature for 24 hours. A slightly opalescent extract was obtained after filtration on a Büchner funnel. The activity of this extract which was stored in a refrigerator at 0° remained intact for a considerable time.

Buffers. Walpole's acetic acid-acetate buffer was used for the acid range, veronal buffer of Michaelis for the p_H range 6.8–9.6 and Sørensen's glycine buffer for the p_H range 8.5–12.9. All solutions were adjusted to the desired p_H before mixing and the p_H of each mixture was determined by the capillator method.

Substrates. The substrates employed were Merck's sodium β -glycerophosphate and sodium hexosediphosphate.

The sodium hexosediphosphate solution was prepared from a pure sample of calcium hexosediphosphate obtained from Messrs British Drug Houses Ltd. A weighed amount was dissolved in water, treated with slightly less than the equivalent amount of pure sodium oxalate and centrifuged. After testing to ensure freedom from oxalate the solution was stored at 0°; fresh solutions were made up at frequent intervals.

Procedure. The hydrolysis was carried out as follows. A series of tubes was set up each containing 5 ml. of substrate (2% sodium glycerophosphate

solution, adjusted to the desired p_H), 10 ml. buffer, 5 ml. of the enzyme solution and water to make up the total volume to 25 ml. When other solutions such as $MgCl_2$, $6H_2O$ were added simple adjustments of the volume of buffer were made, the concentration being kept constant. Control tubes containing enzyme, added ions and buffer were incubated with the experimental tubes. It was found that in the control tubes containing the substrates no detectable amounts of inorganic P were present at any time.

The hydrolysis was carried out in a thermostat at 35° for exactly 4 hours. An aliquot portion of the reaction mixture was then removed, an equal volume of 10 % trichloroacetic acid added and the contents were filtered after 15 min. The filtrate was then analysed for inorganic phosphorus by a colorimetric method [Fiske and Subbarow, 1925]. The increase in inorganic P after deducting the control expressed in mg. P per 10 ml. of reaction mixture measures the activity of the enzyme.

Brain phosphatase activity and hydrogen ion concentration.

The activity of brain phosphatase at various hydrogen ion concentrations is plotted in Fig. 1. The brain extract is found to exhibit two optima for phosphatase activity, one at p_H 9.4–9.6 and the other at p_H 5.0. In this respect

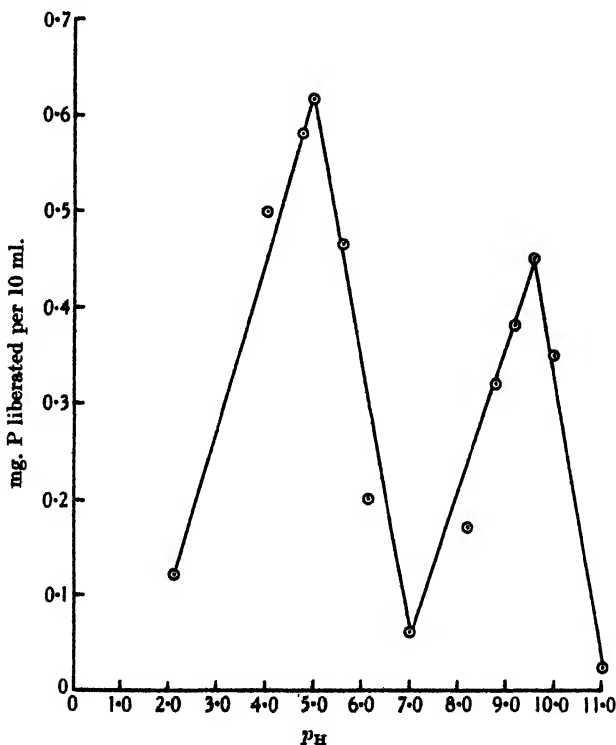


Fig. 1. Effect of p_H on the hydrolysis of sodium β -glycerophosphate by the brain phosphatase.

brain phosphatase resembles spleen, liver and kidney phosphatases, which have been shown by Davies [1934] and Bamann and Riedel [1934] respectively, to contain both alkaline and acid phosphatases.

Presence of two phosphatases in brain.

Sheep's brain was found to contain appreciable quantities of both the acid and alkaline phosphatases. In Table I are given the activities at p_H 5.0 and 9.4 of both the phosphatases of several brain extracts prepared from acetone-treated brain. The ratio of the two phosphatases $\frac{\text{acid phosphatase } (p_H \text{ 5.0})}{\text{alkaline phosphatase } (p_H \text{ 9.4})}$ was very nearly the same in most of the brains examined except in two cases where a much higher value was obtained. Further, the results show that the individual variation in the activity of the phosphatases, particularly the acid phosphatase, is not considerable, so that the quantitative variation in the phosphatase activity of brain under normal and abnormal conditions could be studied without any serious error.

Table I. *The relative activities of the acid and alkaline phosphatases of brain.*

Brain No.	Activity (mg. P in 10 ml. of reaction mixture)		Ratio Activity of the acid phosphatase (p_H 5.0) Activity of the alkaline phosphatase (p_H 9.4)
	Acid phosphatase	Alkaline phosphatase	
1	0.460	0.348	1.32
2	0.460	0.350	1.31
3	0.542	0.404	1.34
4	0.428	0.396	1.10
5	0.378	0.244	1.50
6	0.390	0.284	1.37
7	0.534	0.176	3.00
8	0.520	0.186	3.30

The activity of brain phosphatase as compared with the other tissue phosphatases.

Brain, liver and kidney of the same animal (sheep) were similarly treated with acetone and ether and dried in a desiccator. The dry powders were extracted with water in the same manner as described for brain. The activities of the extracts were determined and the results are presented in Table II.

Table II. *Phosphatase activities of brain, liver and kidney.*

Tissue	The weight of fresh tissue taken for acetone treatment (g.)	The weight of the dry powder obtained after acetone treatment (g.)	Activity (mg. P in 10 ml. reaction mixture after 4 hours' hydrolysis)	
			p_H 9.2	p_H 5.0
Brain	85	12	0.244	0.378
Liver	80	23	0.348	—
Kidney	65	12	0.994	—

Purification of the enzyme.

The crude extract from the dry fat-free brain powder can be purified by the following methods.

Isoelectric precipitation of the inert material at p_H 4.8. Purification was effected by isoelectric precipitation of the inert material at p_H 4.8 by adding 0.2M acetic acid-acetate buffer to three times the volume of the extract and centrifuging after keeping the mixture at 0° for 30 min. A water clear extract obtained in this manner was found to be quite active. The extract can be further purified by dialysis in a collodion bag for 24 hours.

Dialysis. Short-period dialysis (24 hours) of the crude extract removed the phosphate almost completely without any loss of activity of the enzyme. On continuing the dialysis for about 15 days however the activity was almost completely lost and could be regained by addition of magnesium.

Ultrafiltration. Purification of the extract by high pressure ultrafiltration was found to be quite satisfactory. Ultrafiltration was carried out through cellophane sheet No. 300 under a pressure of 70 kg. per cm.² The extract was kept stirred electromagnetically during filtration. This prevented clogging of the membrane and facilitated rapid filtration. The cellophane sheet was soaked in water for 24 hours before use. Under these conditions the filtration was effected in a very short time about 10–15 ml. being filtered in 1 hour. The residue obtained after the ultrafiltration was found to have retained intact the activity of the acid phosphatase and to have lost almost completely the activity of the alkaline phosphatase which however could be restored by addition of the ultrafiltrate. This method affords a very convenient means of obtaining a preparation of the acid phosphatase only.

The results of a typical experiment are shown in Table III. 40 ml. of the crude extract were filtered and the residue on the filter after complete filtration was removed by washing repeatedly with distilled water and made up to the original volume. The activities of the alkaline and acid phosphatases of the ultrafiltered enzyme extract thus obtained were determined in presence and absence of the ultrafiltrate. For activity determination 5 ml. of the ultrafiltered enzyme extract and 5 ml. of the ultrafiltrate were used.

Table III. *Ultrafiltration of the aqueous extract of acetone-treated brain.*

	Activity (mg. P in 10 ml. of reaction mixture)	
	Alkaline phosphatase (p_H 9.2)	Acid phosphatase (p_H 5.0)
1. Ultrafiltered enzyme	0.02	0.153
2. Ultrafiltrate	0	0
3. Ultrafiltered enzyme plus ultrafiltrate	0.178	0.158

Acetone precipitation. The enzyme was also obtained in the form of a dry powder by precipitation from the aqueous extract with twice the volume of acetone after isoelectric precipitation of the impurities. The precipitate was separated by centrifuging, washed with absolute alcohol and ether and dried in a desiccator. A white powder was obtained which was found to exhibit very little activity of the alkaline phosphatase; activity could be restored by addition of magnesium. The preparation retained however the activity of the acid phosphatase.

Effect of magnesium on brain phosphatase.

The influence of magnesium on the activity of brain phosphatase was studied by using both dialysed and undialysed extracts of the acetone-treated brain. Dialysis of the crude extract was carried out in collodion bags against distilled water for 3 days. The activity of the alkaline phosphatase of both crude and dialysed extracts in presence of varying concentrations of $MgCl_2 \cdot 6H_2O$ was determined at p_H 9.4 at 35°. The results are shown in Table IV.

It is evident that the percentage activation of the phosphatase by magnesium is greater in dialysed preparations. Thus an increase of activity of about 600 % is shown by the dialysed extract, whilst the activity of the crude extract increased

Table IV. *Effect of magnesium on the activity of brain phosphatase*

Added Mg concentration (<i>M</i>)	Crude extract		Dialysed extract	
	Activity (mg. P in 10 ml. of reaction mixture)	Activation %	Activity (mg. P in 10 ml. of reaction mixture)	Activation %
0	0.127	—	0.083	—
0.0004	0.222	75	0.412	395
0.001	0.264	108	0.506	510
0.002	0.258	103	0.555	568
0.010	0.228	80	0.606	628
0.020	—	—	0.597	618
0.040	—	—	0.588	607

by only 100% at optimum concentration of magnesium. The concentration of added magnesium for maximum activation of dialysed enzyme extract is found to lie between 0.01 and 0.02 *M*, while for the crude extract it is only 0.001–0.002 *M*. This is in conformity with the observations of Jenner and Kay [1931]. A possible explanation of this finding is that probably the crude extract contains magnesium in an amount to account for partial activation of the enzyme.

The acid phosphatase of the brain extract behaves differently from the alkaline phosphatase towards magnesium. It does not show any increased activity in presence of magnesium at p_H 5.0. It loses much of its activity on dialysis which cannot be restored by the addition of magnesium. That the loss of activity of the acid phosphatase during dialysis is due to autolysis and not to the removal of any activator is shown by the fact that the activity of the dialysed extract cannot be increased by adding boiled brain extract. It appears from these results that the acid phosphatase of brain is identical with the acid phosphatases of liver and spleen which according to Bamann and Riedel [1934] and Davies [1934] respectively are not activated by magnesium.

Time course of the action of the acid and alkaline phosphatases of brain.

The experiment was carried out at 35° using 20 ml. of the enzyme, 20 ml. of 2% sodium glycerophosphate solution, 40 ml. of buffer (acetic acid-acetate buffer of p_H 5.0 and glycine buffer of p_H 9.4 respectively) and sufficient water to bring the volume up to 100 ml. 5 ml. of this solution were analysed at once for P after the enzyme had been added; subsequent samples (5 ml.) were analysed for P at known intervals of time. The rate of hydrolysis of sodium glycerophosphate by extracts of acetone-treated brain at p_H 5.0 and 9.4 are plotted in Fig. 2. The broken lines indicate the time course of the action of dialysed extracts with and without the addition of magnesium. All the curves can be considered to be linear till about 10–12% of the substrate is hydrolysed. Subsequently the rate of reaction diminishes. The course of the action of the phosphatase is therefore of zero order. For reaction times up to 6 hours the amount of hydrolysis affords a good measure of the initial reaction velocity. 4-hour period hydrolysis was therefore adopted for all experiments on the determination of the activity of the enzyme.

Relation between substrate concentration and brain phosphatase activity.

According to Michaelis and Menten [1913] an enzyme combines with its substrate to form a compound and the speed of the reaction depends directly upon the concentration of the enzyme-substrate compound. The theory which

was originally founded on experiments with the invertase-sucrose system has been extended to other enzyme systems. To decide whether the concentration of the enzyme-substrate compound determines the rate of the reaction, it is

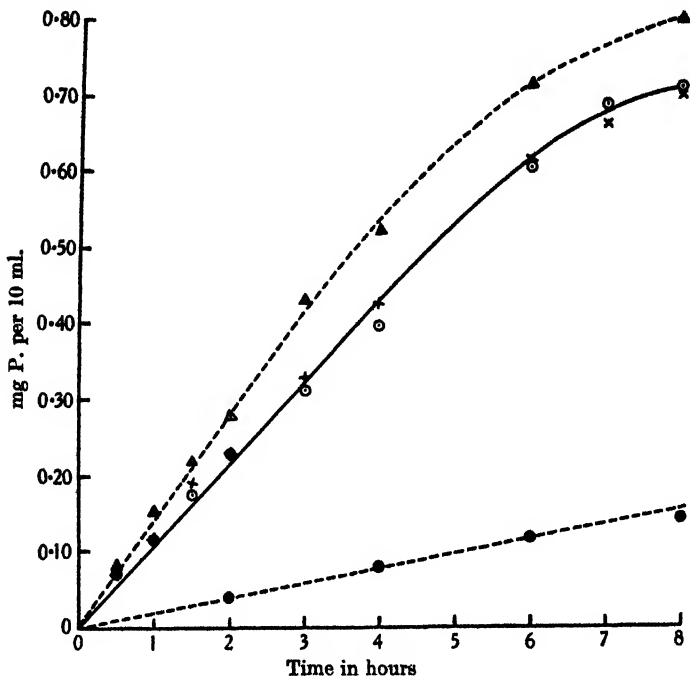


Fig. 2. Rate of hydrolysis of glycerophosphate, by the brain phosphatase.

- Alkaline phosphatase (undialysed).
- ×—× Acid phosphatase (undialysed).
- Alkaline phosphatase (dialysed).
- △—△ Alkaline phosphatase (dialysed) activated by 0.01 *M* Mg⁺⁺.

necessary to examine the applicability of Michaelis and Menten's equations to the enzyme system concerned. The observed initial reaction velocity v is given by the equation (*cf.* Michaelis and Menten)

$$v = V_{\max} (S) / [K_s + (S)],$$

where v is the initial velocity of hydrolysis. V_{\max} is a numerical constant representing the maximum velocity obtained when the enzyme E exists completely in the form ES [$V_{\max} = K (E_{\text{total}})$]. S is the substrate concentration and K_s (the Michaelis constant) is the dissociation constant of the enzyme-substrate compound. The value of the dissociation constant is given by the substrate concentration at half-maximum velocity. In order to decide whether combination between enzyme and substrate according to the mass law actually takes place the calculation of the substrate concentration at half-maximum velocity is not enough. Further examination of the results is essential to show that the enzyme system concerned behaves according to Michaelis and Menten's theory. The possibility of the application of this theory to brain phosphatase was tested. The constants V_{\max} and K_s were evolved by the graphical method described by Lineweaver and Burk [1934].

The activity of brain phosphatase prepared by extraction of the acetone-treated brain was determined in the presence of substrate concentrations varying from 0.0032 to 0.640 *M* at 35°. The p_H of each digest was adjusted to 9.2 and 5.0 for alkaline and acid phosphatases respectively. The activities of the two phosphatases were determined by the procedure already described. Typical results are presented in Table V.

Table V. *Relation between substrate concentration and brain phosphatase activity.*

Glycerophosphate concentration (<i>S</i>) <i>M</i>	Alkaline phosphatase activity at p_H 9.2		Acid phosphatase activity at p_H 5.0	
	Observed mg. P per 10 ml. reaction mixture after 4 hours' hydrolysis	Calculated from the equation $v = \frac{0.66(S)}{(S) + 0.012}$	Observed mg. P per 10 ml. reaction mixture after 4 hours' hydrolysis	Calculated from the equation $v = \frac{1.66(S)}{(S) + 0.029}$
0.0032	0.134	0.139	0.178	0.165
0.0064	0.230	0.229	0.300	0.300
0.0128	0.350	0.341	0.504	0.508
0.032	0.430	0.480	0.766	0.860
0.064	0.350	0.555	1.120	1.140
0.128	0.290	0.603	1.142	1.360
0.320	0.280	0.639	1.620	1.520
0.640	—	—	0.992	1.590

It is evident that there is a close agreement between the observed and calculated values. The initial velocity of hydrolysis can therefore be predicted by the theory that glycerophosphate enters into combination with the phosphatase. Recently Folley and Kay [1935] have shown that the phosphatases of kidney and mammary gland behave in accordance with the theory of Michaelis and Menten, the Michaelis constant being the same for each. In the present case, the two phosphatases of brain differ in their behaviour towards the substrate the Michaelis constants of the two phosphatases being different; 0.012 was obtained for the alkaline phosphatase, and 0.029 for the acid phosphatase showing that the latter has less affinity for the substrate than the former. Further the results indicate that in the range of concentration above 0.032 *M* and 0.320 *M*, for alkaline and acid phosphatases respectively, the observed velocity was always less than the calculated. This is possibly due to a decrease in the relative water concentration. Further work is however necessary to elucidate this point.

Action on different substrates.

The hydrolytic activities of the two phosphatases of brain on sodium β -glycerophosphate and sodium hexosediphosphate were next examined. In each test the enzyme was allowed to act on an amount of ester equivalent to 10 mg. P at 35° and at the optimum p_H for the enzyme. The total P was determined by the method of King [1932].

Table VI. *Action on different substrates.*

Substrate	Activity of the alkaline phosphatase at p_H 9.2 mg. P in 10 ml. of reaction mixture	Activity of the acid phosphatase at p_H 5.0 mg. P in 10 ml. of reaction mixture
Sodium glycerophosphate	0.174	0.215
Sodium hexosediphosphate	0.240	0.310

The results show that sodium hexosediphosphate is more easily hydrolysed than sodium β -glycerophosphate by the alkaline and acid phosphatases of brain.

SUMMARY.

1. Brain phosphatase exhibits two optima: one at p_H 9.4-9.6 and the other at p_H 5.0.
2. The ratio between the activities of the two phosphatases is almost constant for brains of animals of the same species.
3. The activity of acetone-treated brain extracts is about as high as that of liver of the same animal.
4. The phosphatase can be purified by (a) isoelectric precipitation of the inert material at p_H 4.8, (b) ordinary dialysis, (c) ultrafiltration through cellophane membrane and (d) acetone precipitation.
5. Magnesium ions activate the alkaline phosphatase while the acid phosphatase remains unaffected. The percentage activation is considerably enhanced when the enzyme extract has been previously purified by dialysis.
6. The time course of the reaction of the alkaline and acid phosphatases is linear in character in the early stage of hydrolysis irrespective of the purity of the enzyme and presence or absence of magnesium.
7. The initial rate of hydrolysis by the alkaline and acid phosphatases varies with the substrate concentration in a manner predictable by the theory of Michaelis and Menten.
8. Sodium hexosediphosphate is more easily hydrolysed than sodium glycerophosphate by the two phosphatases.

Our thanks are due to the Lady Tata Memorial Trust for the award of a scholarship to one of us (N. C. D.). Thanks are also due to Prof. Subrahmanyam for his keen interest in the present investigation.

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CLVIII. ENZYMES IN CANCER.

II. THE GLYCEROPHOSPHATASES OF HUMAN ERYTHROCYTES.

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THE β -glycerophosphatase activity of erythrocytes from human cancer patients has been found to be frequently higher than that of erythrocytes from normal subjects, whilst the absolute increase in activity when magnesium ions were added appeared to be the same in both instances [Schoonover and Ely, 1935]. Therefore, the portion of the cancer erythrocyte β -glycerophosphatase which furnished the increase over the normal amount seemed to be incapable of magnesium activation.

There exists in animal organs [Davies, 1934; Bamann and Riedel, 1934] an enzyme which could be responsible for this phenomenon, and it may conceivably occur in the red blood cells. On the other hand, it is also possible that peculiar conditions of natural activation could be the cause.

A comparison of the erythrocyte type of phosphoesterase and the organ type of phosphoesterase shows that their most outstanding similarity lies in their acid p_H optima. The erythrocyte phosphatase [Davies, 1934] as well as the phosphatases of bottom yeast [Schäffner and Bauer, 1935] and of *Propionibacterium jensenii* [Pett and Wynne, 1933] hydrolyse α -glycerophosphate more rapidly than the β -isomeride and are readily activated by magnesium ions. The organ phosphatase [Davies, 1934; Bamann and Riedel, 1934], like the acid phosphatases of urine [Kutscher, 1935], of top yeast [Albers and Albers, 1934, 2] and of *Clostridium acetobutylicum* [Pett and Wynne, 1933], prefers the β -ester and is seldom activated by magnesium at or below its optimum p_H . With lower hydrogen ion concentrations, the organ enzyme shows activation; the others are inhibited. It has been suggested by Albers and Albers [1934, 1] that this activation is due to imperfect removal of erythrocytes from the enzyme preparations.

Phosphatase activity may be augmented by materials other than magnesium ions. Jenner and Kay [1931] found that phosphoesterases from various sources, especially from the red blood cells, retain a large part of their activity when freed from all but traces of magnesium. Moreover, erythrocyte phosphatase was sometimes found to be activated by magnesium ions. Bamann, Riedel and Diederichs [1934] have shown that the capacity for magnesium activation of the alkaline phosphatase is a function of the age and purity of the enzyme preparation and also of the solubility of the enzyme complex.

In an attempt to define the origin of the behaviour of cancer erythrocytes towards β -glycerophosphatase, some experiments were made with the α - and β -glycerophosphatase activities of the haemolysates of normal and of cancerous human erythrocytes. In accord with the theory that the organ enzyme, which

prefers the β -ester, is present in large amounts in cancer erythrocytes, no difference was found between the average α -glycerophosphatase activities of normal and of cancer erythrocytes. The weight of the evidence, however, makes it unlikely that this theory is correct. Both α - and β -glycerophosphatase showed quite similar behaviour with regard to magnesium activation. Artificial conditions produced a destruction of the capacity of both glycerophosphatases for magnesium activation, although considerable dephosphorylating power towards both substrates persisted. α -Glycerophosphatase activity exhibits considerable variation from one subject to another; it is the β -, and not the α -glycerophosphatase activity, which appears to be the more constant in the normal individual. The peculiarities observed in the β -glycerophosphatase activity of cancerous erythrocytes, then, are a distinct departure from the normal state, and make it possible that other phosphoric acid esters also may be hydrolysed in some unusual manner.

EXPERIMENTAL PROCEDURE.

The erythrocyte samples used in this investigation were obtained from a new series of cancer patients and normal individuals; only two of the latter have furnished data previously. It was not always possible to secure patients whose malignant condition had advanced as far as it had in the series reported previously [Schoonover and Ely, 1935].

The buffered substrates were made up daily until it became apparent that new lots of chemicals affected the p_H of these solutions to an undesirable extent. Later, they were made up in quantity and kept under the conditions recommended by Bodansky [1933] for the preservation of the more alkaline substrates. Quinhydrone p_H determinations at room temperature were conducted on each buffered substrate. There was no appreciable change in p_H , nor was any formation of inorganic phosphate found, over an interval of 3 weeks. The phosphatase units (mg. of inorganic phosphorus liberated by 10 ml. of erythrocytes in 24 hours at 37°) observed were plotted against the p_H values of the buffered substrates; from these p_H -activity curves the value for any desired p_H within the range could be read off.

Buffered substrates containing β -glycerophosphate were made up in the proportions described before and covered the approximate p_H range 5.0–6.0 at intervals of about 0.2 p_H . Buffered substrates containing α -glycerophosphate were prepared from a fresh stock solution of sodium α -glycerophosphate and sodium acetate, which was obtained in the following fashion: to 100 ml. of 0.6 *M* calcium α -glycerophosphate (neutral, DAB 6, Fraenkel and Landau) in *N* acetic acid there were added 4.5 ml. of water, and then, with shaking, 95.5 ml.¹ of 0.6 *M* oxalic acid and 240 ml. of *N* NaOH. After 10–15 min., the mixture was filtered, and 300 ml. of the filtrate, together with 35.4 g. of anhydrous sodium acetate, were made up to 500 ml. with water. Two ml. of this solution, diluted to 10 ml., as in the reaction mixture, contain an amount of phosphorus corresponding closely to that of the β -glycerophosphate substrates; moreover, the 10 ml. are 0.2 *M* in acetate ion, also as in the β -glycerophosphate substrates. The individual substrate solutions, then, were prepared by diluting 2 vols. of the sodium α -glycerophosphate-sodium acetate solution to 5 vols. with *M* magnesium chloride (when magnesium was to be added to the reaction mixture),

¹ 95.5 ml. was the quantity selected because the precipitate of calcium oxalate, after the addition of NaOH, corresponded to 95.4 ml. of oxalic acid. When 100 ml., the theoretical quantity, was employed, the calcium oxalate precipitate corresponded to only 95.7 ml. of oxalic acid.

0.5*N* HCl in sufficient quantity to produce the p_H required and water. The p_H range 5.0–6.0, at intervals of about 0.2 p_H , was covered in this way.

To 5 ml. of each β -glycerophosphate substrate, after 5 min. previous heating at 37°, were added 5 ml. of a 1 : 50 haemolysate of unwashed erythrocytes. The remaining haemolysate was diluted with an equal volume of water, and after mixing, 5 ml. of this 1 : 100 dilution were added to each substrate containing α -glycerophosphate. This procedure was rendered necessary by the extremely rapid cleavage of α -glycerophosphate by erythrocytes. The rest of the experimental procedure was exactly the same as that described in the previous paper. The use of the Bodansky [1932] table and factors to allow for the effect of Beer's law and for the influence of β -glycerophosphate and trichloroacetic acid on the colorimetric readings was found to be advantageous when the α -ester had replaced the β -ester.

p_H optimum for erythrocyte α -glycerophosphatase activity in the range 5.0–6.0.

Fig. 1 shows that the optimum substrate p_H for the hydrolysis of α -glycerophosphate by normal and by cancerous human erythrocytes is found to be close

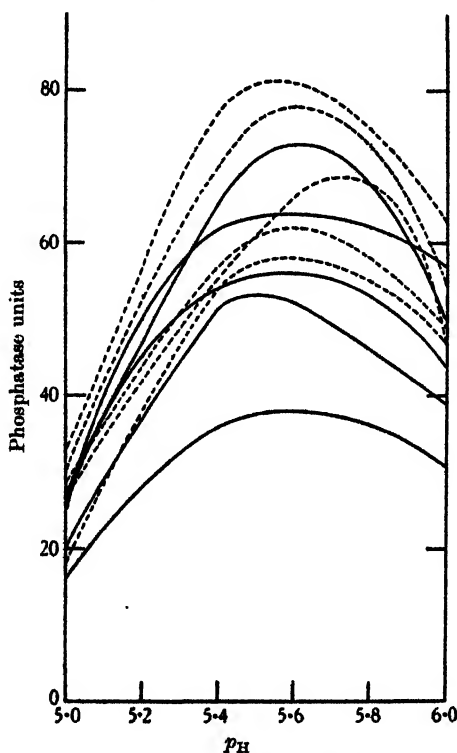


Fig. 1. p_H -activity curves for the α -glycerophosphatase activity of human erythrocytes. — normal subjects; - - - - - untreated cancer patients.

to 5.6 under the experimental conditions described. This is the same optimum as that for the hydrolysis of β -glycerophosphate.

Relation of α - to β -glycerophosphatase activity.

In Table I may be seen examples of the rates of hydrolysis of α - and β -glycerophosphates by red blood cell haemolysates from normal and cancerous individuals. α -Glycerophosphate is split by both haemolysates at rates which vary considerably from one subject to another. The cancer α -glycerophosphatase figures fall within the normal limits. Half of the cancer β -glycerophosphatase values, on the other hand, lie above the highest normal figure. The low values observed in the other half of the cancer cases are possibly due to the slighter degree of advancement in the malignant process. An examination of the ratios of the rates of hydrolysis of the two isomerides shows that there is no constant ratio in either the normal or the cancerous state; in fact, there appears to be an independence of submission to enzymic attack.

Table I. *Comparison of the rates of hydrolysis of α - and β -glycerophosphates by erythrocytes from the same individuals.*

No.	Type	p_H 5.0			p_H 5.6			p_H 6.0		
		α -Gly- cero- phos- phate	β -Gly- cero- phos- phate	α/β	α -Gly- cero- phos- phate	β -Gly- cero- phos- phate	α/β	α -Gly- cero- phos- phate	β -Gly- cero- phos- phate	α/β
		Units	Units		Units	Units		Units	Units	
1	Normal	16	4.1	3.9	38	8.4	4.5	31	7.7	4.0
2	"	20	4.0	5.0	52	8.7	6.0	39	7.5	5.2
3	"	33	5.7	5.8	54	11.2	4.8	37	11.2	3.3
4	"	26	5.2	5.0	56	9.2	6.1	44	7.2	6.1
5	"	39	6.4	6.1	62	8.9	7.0	38	—	—
6	"	25	5.1	4.9	64	9.9	6.5	57	8.8	6.5
7	"	27	5.4	5.0	65	10.4	6.3	51	10.3	5.0
8	"	27	6.4	4.2	73	11.9	6.1	50	9.8	5.1
9	"	45	7.1	6.3	84	11.9	7.1	70	12.9	5.4
1	Cancer	22	6.2	3.6	44	10.9	4.0	—	—	—
2	"	19	5.2	3.7	57	9.5	6.0	45	8.5	5.3
3	"	39	19.5	2.0	57	20.2	2.8	36	19.0	1.9
4	"	18	4.7	3.8	58	9.7	6.0	47	8.8	5.3
5	"	38	10.1	3.8	61	15.5	3.9	47	14.2	3.3
6	"	28	5.3	5.3	62	10.3	6.0	49	9.7	5.1
7	"	26	6.2	4.2	66	11.4	5.8	47	10.2	4.6
8	"	46	10.2	4.5	69	19.6	3.5	55	15.2	3.6
9	"	30	7.2	4.2	78	13.0	6.0	55	10.8	5.1
10	"	33	7.7	4.3	81	13.4	6.0	63	12.0	5.3

Activation of α - and β -glycerophosphatases by magnesium.

Erythrocyte α -glycerophosphatase activity, like erythrocyte β -glycerophosphatase activity, is augmented by the presence of magnesium chloride. According to the preliminary data recorded in Table II, 0.02 *M* magnesium

Table II. *Effect of magnesium chloride in large concentrations on the α -glycerophosphatase activity of normal and cancer erythrocytes.*

p_H	Normal				Cancer			
	<i>M</i> MgCl ₂ added to reaction mixture				<i>M</i> MgCl ₂ added to reaction mixture			
	0	0.02	0.05	0.10	0	0.02	0.05	0.10
	Units	Units	Units	Units	Units	Units	Units	Units
5.0	27	46	47	43	31	48	51	43
5.6	65	95	84	61	60	97	88	62
6.0	51	82	67	37	50	—	—	—
6.2	31	—	—	—	37	75	56	27

chloride appears to be the most nearly favourable concentration of the three tried for effecting complete activation.

In subsequent studies, when sufficient material was available, an extensive survey was made of the effects of magnesium chloride upon the rate of cleavage of both phosphoric acid esters. In others, where experiments could be carried out only under selected conditions, the results are reported by average. The increases produced in the actions of normal and cancer erythrocyte α - and β -glycerophosphatase are presented in Table III.

The qualitative effect of magnesium chloride upon the hydrolysis of the two substrates is much the same. That is, the increase with low concentrations of magnesium chloride tends to become greater as the p_H approaches that of neutrality, actually resulting in a displacement of the p_H optimum. The best examples for comparison are found in the cases of α -glycerophosphate in the presence of 0.01 M magnesium chloride and of β -glycerophosphate in the presence of 0.02 M magnesium chloride. This relation is to be expected, since the amount of enzyme source added to the α -glycerophosphate solution is one-half that added to the β -glycerophosphate solution.

Cancerous erythrocytes appear to respond less readily, in general, to magnesium stimulation than do normal erythrocytes. In the former communication [Schoonover and Ely, 1935] it was stated that the increases in β -glycerophosphatase activity produced by magnesium in cancer red blood cells were usually equal to those found in normal red blood cells. However, the cases which are reported in full in Table III represent those which displayed β -glycerophosphatase activities within or only slightly above the normal range (see Table I). Therefore, the previous observation that cancer erythrocyte β -glycerophosphatase is capable of less proportionate, or percentage, activation by magnesium still retains its validity. This finding is borne out by the averages of scattered values, where the cancer average now includes cases with higher β -glycerophosphatase activities as well. I have not been able, unfortunately, to observe the tremendous activation observed formerly [Schoonover and Ely, 1935] when 0.1 M magnesium

Table III. *Unit increases produced in α - and β -glycerophosphatase activities of normal and cancerous human erythrocytes by the addition of various concentrations of magnesium chloride to the reaction mixture.*

No. from Table I	Type	p_H 5.0						p_H 5.6						p_H 6.0					
		α M $MgCl_2$		β M $MgCl_2$				α M $MgCl_2$		β M $MgCl_2$				α M $MgCl_2$		β M $MgCl_2$			
		0.01	0.02	0.02	0.05	0.10		0.01	0.02	0.02	0.05	0.10		0.01	0.02	0.02	0.05	0.10	
1	Normal	3	8	6.4	9.3	8.2	28	24	17.1	18.4	14.6	31	23	21.5	19.1	16.6			
2	"	3	1	6.9	8.1	6.8	20	24	14.0	17.1	11.1	35	29	19.0	19.2	13.1			
4	"	-1	10	4.9	7.4	6.8	21	22	14.8	15.8	10.4	24	23	21.3	18.9	8.8			
6	"	12	16	5.9	7.9	7.9	29	24	13.8	15.1	10.6	23	10	18.3	16.2	7.0			
8	"	10	14	5.9	9.0	8.8	22	26	17.6	18.0	10.0	29	20	22.1	17.2	8.0			
2	Cancer	12	7	4.9	7.8	5.6	17	14	13.6	14.7	5.7	15	9	17.1	13.3	3.8			
4	"	13	8	6.0	7.6	7.7	17	3	14.0	14.8	9.0	17	6	18.9	15.4	6.7			
6	"	-7	5	4.3	5.9	5.7	11	14	11.9	12.3	6.2	16	9	15.5	12.4	4.5			
7	"	5	7	5.9	8.2	6.9	19	22	13.8	15.4	10.2	—	24	18.4	14.6	6.5			
9	"	5	7	3.0	5.1	3.7	12	10	10.1	10.9	3.6	18	13	15.2	12.5	3.4			
Normal average		5	10	6.0	8.3	7.7	24	24	15.5	16.9	11.3	28	21	20.4	18.1	10.7			
Cancer average		6	7	4.8	6.9	5.9	15	13	12.7	13.6	6.9	17	12	17.0	13.6	5.0			
Average for all subjects from Table I including above:																			
Normal average		—	12	—	—	7.6	—	28	—	16.6	—	—	27	19.1	—	—			
Cancer average		—	10	—	—	6.7	—	25	—	14.8	—	—	24	18.6	—	—			

chloride was added to the β -glycerophosphate reaction mixtures of p_H 5.0 which contained erythrocytes from normal male subjects. This may be due to slight variations in the hydrogen ion concentrations of the earlier buffered substrates, for the p_H -activity curve rises sharply in the region of p_H 5.0. The results, consequently, show no sex difference and hence are no longer segregated according to sex.

Further examination of the relative effects of the most nearly optimum magnesium chloride concentrations, i.e. the lower ones, upon the hydrolysis of the two esters reveals a noteworthy result. The increase in α -glycerophosphatase activity compared with that in β -glycerophosphatase activity is somewhat greater in terms of mg. of inorganic phosphorus released. The increase is, however, not nearly as great as might be expected from the ratios of the rates of hydrolysis of the two glycerophosphates when no magnesium has been added to the reaction mixture.

Constancy of α - and β -glycerophosphatase activities in vivo and in vitro.

The β -glycerophosphatase activity of erythrocytes from the same normal subjects has been studied at different times. Table IV illustrates the constancy of this enzymic ability in the normal individual, both in its state of natural activation and in its response to magnesium stimulation. There appears to be no seasonal variation in β -glycerophosphatase activity. Several normal subjects studied during the summer yielded results within the limits imposed by data obtained from other normal subjects during the winter. Nos. 6 and 7 in Table IV furnish examples in which there are available for comparison figures obtained from the same subjects at other seasons.

Table IV. *Constancy of β -glycerophosphatase activity in the normal individual.*

No.	Date	p_H 5.0				p_H 5.6				p_H 6.0			
		Units	Units with 0.10 M	Unit in-crease	% in-crease	Units	Units with 0.05 M	Unit in-crease	% in-crease	Units	Units with 0.02 M	Unit in-crease	% in-crease
		MgCl ₂	MgCl ₂	crease	crease	MgCl ₂	MgCl ₂	crease	crease	MgCl ₂	MgCl ₂	crease	crease
1	Jan. 11	3.1	12.7	9.6	301	6.4	24.9	18.5	289	—	—	—	—
	Feb. 21	2.3	11.9	9.6	417	5.9	25.7	19.8	334	—	—	—	—
2	Jan. 15	5.7	20.2	14.5	254	13.4	30.8	17.4	130	12.2	—	—	—
	May 16	5.7	12.6	6.9	121	11.2	27.2	16.0	143	11.2	—	—	—
3	Feb. 14	5.2	12.4	7.2	138	10.0	25.5	15.5	155	10.0	25.7	15.7	157
	Apr. 23	5.4	11.5	6.1	115	10.4	26.2	15.8	152	10.3	27.9	17.6	171
4	Feb. 20	4.7	—	—	—	9.3	—	—	—	—	—	—	—
	Oct. 8	4.3	—	—	—	8.9	—	—	—	—	—	—	—
5	Mar. 7	4.4	13.1	8.7	198	8.5	25.1	16.6	195	8.3	23.8	20.5	247
	Apr. 30	4.4	12.4	8.0	182	8.8	24.3	15.6	177	8.1	27.3	19.2	237
6	May 13	7.1	15.1	8.0	113	11.9	28.8	16.9	142	12.9	29.8	16.9	131
	July 15	6.0	12.9	6.9	115	11.3	29.7	18.4	163	10.6	28.9	18.3	173
7	June 26	6.9	14.7	7.8	113	13.1	31.5	18.4	140	11.3	34.5	23.2	205
	Oct. 31	6.4	15.2	8.8	138	11.9	29.9	18.0	151	9.8	31.9	22.1	226

The α -glycerophosphatase activity of both normal and cancer erythrocytes is not so constant. It decreased remarkably in summer, although magnesium chloride was still able to exert its usual influence and sometimes an even greater influence. This statement is derived from the averages contained in Table V, which may be compared with those in Tables I and III. The summer alteration in the α -glycerophosphatase activity forms a close parallel with the apparent alteration in α -glycerophosphatase when only one half the usual amount of

enzyme source is present in the reaction mixture. Thus, it may be attributed to the dilution of a natural activator. (Whilst identical figures, calculated as mg. of inorganic phosphorus liberated by 10 ml. of erythrocytes, were obtained by the use of either 0.10 or 0.05 ml. of erythrocytes, the use of 0.025 ml. resulted in the data to be seen in Table V.)

Table V. *α -Glycerophosphatase activities of normal and cancer erythrocytes in summer and in reaction mixtures containing one half the usual amount of enzyme source.*

	p_H 5.0				p_H 5.6				p_H 6.0			
	Units with 0.02 M		Unit in-	% in-	Units with 0.02 M		Unit in-	% in-	Units with 0.02 M		Unit in-	% in-
	Units	MgCl ₂	crease	crease	Units	MgCl ₂	crease	crease	Units	MgCl ₂	crease	crease
Summer average:												
Normal (5)	19	39	20	146	33	79	46	237	19	65	46	352
Cancer (4)	33	48	15	47	40	75	35	91	13	60	47	409
Normal No. 6 from Table IV:												
May 13	45	60	15	33	84	113	29	35	70	99	29	41
July 15	33	56	23	70	69	97	28	41	21	59	38	181
Normal No. 7 from Table IV:												
Oct. 31	27	41	14	52	73	99	26	36	50	70	20	40
June 26	33	57	24	73	49	93	44	90	30	68	38	127
One half the usual amount of enzyme source (average):												
Normal (2)	11	31	20	210	17	55	38	225	10	51	41	491
Cancer (2)	25	45	20	82	44	79	35	93	24	63	39	182

Table VI. *Effect of preservation on the β - and α -glycerophosphatase activities of haemolysed and unhaemolysed erythrocytes from normal males.*

	Date	p_H	β -Glycerophosphatase M MgCl ₂ added				α -Glycerophosphatase M MgCl ₂ added		
			0	0.02	0.05	0.10	0	0.01	0.02
			Units	Units	Units	Units	Units	Units	Units
Haemolysed red blood cells from a normal male subject	Oct. 14	5.2	—	—	—	—	31	51	55
	" 24	—	—	—	—	—	32	28	33
	" 14	5.6	8.7	23.8	25.3	18.8	—	—	—
	" 24	—	8.5	18.3	22.5	18.0	—	—	—
	" 14	5.8	—	—	—	—	47	77	76
	" 24	—	—	—	—	—	37	48	48
Unhaemolysed red blood cells from a normal male subject	Oct. 17	5.0	4.2	8.9	12.0	11.8	16	21	25
	" 24	—	2.8	5.7	7.4	9.1	9	15	22
	" 17	5.2	6.3	13.7	16.8	16.6	—	—	—
	" 24	—	5.2	10.7	13.3	14.6	—	—	—
	" 17	5.4	7.3	18.8	21.1	19.2	—	53	52
	" 24	—	7.7	16.4	20.4	18.5	—	58	62
	" 17	5.6	—	—	—	—	—	62	—
	" 24	—	—	—	—	—	—	73	—
	" 17	5.8	8.2	27.5	25.6	24.5	—	—	—
	" 24	—	10.1	27.0	25.6	19.5	—	—	—
	" 17	6.0	—	—	—	—	33	63	54
	" 24	—	—	—	—	—	48	72	65
	" 17	6.2	8.0	28.9	27.0	13.1	—	—	—
	" 24	—	9.9	28.7	28.1	12.2	—	—	—

Table V contains a slight inaccuracy. At one time during the summer the hydrogen ion concentrations of the buffered substrates were not checked with sufficient frequency. Within this period, all the α -glycerophosphate reaction mixtures were slightly less acid than they were supposed to be. Therefore, the optimum p_H appeared to shift to the acid side of p_H 5.6. Subsequent experiments, however, still showed the decrease in α -glycerophosphatase activity, together with the practically unchanged response to magnesium chloride, which apparently accompany warm weather.

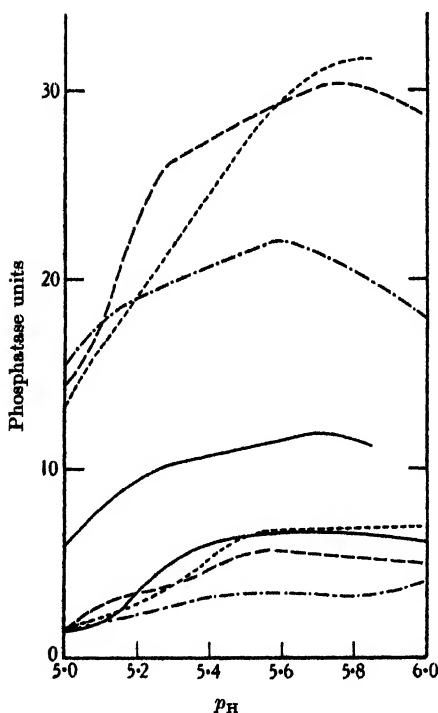


Fig. 2 A.

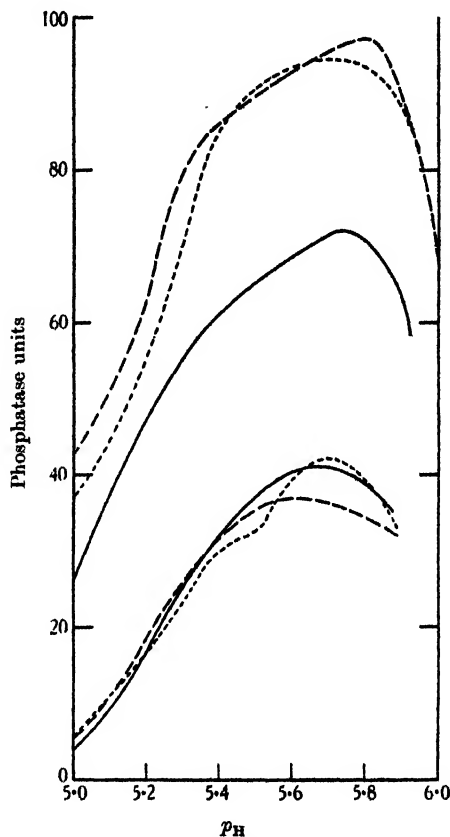


Fig. 2 B.

Fig. 2 A. p_H -activity curves for the β -glycerophosphatase activity of fresh and 19-day old erythrocytes. The four upper curves are from fresh erythrocytes; the four lower curves from 19-day old erythrocytes. — no $MgCl_2$ added to the reaction mixture; 0.02 M $MgCl_2$ added; — — — 0.05 M $MgCl_2$ added; — · — · — 0.10 M $MgCl_2$ added.

Fig. 2 B. p_H -activity curves for the α -glycerophosphatase activity of fresh and 19-day old erythrocytes. The three upper curves are from fresh erythrocytes; the three lower curves from 19-day old erythrocytes. — no $MgCl_2$ added to the reaction mixture; 0.01 M $MgCl_2$ added; — — — 0.02 M $MgCl_2$ added.

This behaviour is not due merely to phosphate inhibition, as might be supposed from the statement of Havard and Reay [1925] that blood phosphorus is increased during the summer months. No especially high inorganic phosphorus content was found in these red blood cell samples. Inhibition of neither

α - nor β -glycerophosphatase activity could in addition be observed when phosphoric acid-sodium hydroxide mixtures of suitable p_H were added to the substrates in amounts representing $1\frac{1}{2}$ –30 times the amount of inorganic phosphorus present in the erythrocyte haemolysates.

The enzyme activity with and without magnesium chloride addition persists for some time in both laked and unlaked red blood cells from normal male subjects, when the samples are kept under toluene in the ice-box. The β -glycerophosphatase system is, however, the more stable. The data are found in Table VI. The studies were made at scattered p_H values, but the substrates were from the same lot as that employed for the fresh samples.

After 19 days in the ice-box under toluene, the intact red cells of a normal male subject lost much of their glycerophosphatase activity. They also lost nearly all of their ability to be activated by magnesium chloride, inhibition being noted under most conditions. The data obtained before and after the 19-day interval may be found in Figs. 2 A and 2 B.

DISCUSSION.

In both normal and cancerous erythrocytes, the α - and β -glycerophosphatase activities show similarity in their p_H optima and in their capacity for magnesium activation over the p_H range 5.0–6.0. The hydrolysis of both glycerophosphates seems to involve two enzymic factors, one of which is activated by magnesium, while the other is not. The first enzymic factor appears to act upon both α - and β -glycerophosphates and is present, on an average, in equal amounts in both normal and cancerous erythrocytes. The existence of the second enzymic factor is made probable by the work of Jenner and Kay [1931], who found activity of erythrocyte phosphatase in the presence of minimum amounts of magnesium, whilst a form of alkaline phosphoesterase incapable of magnesium activation was reported by Bamann *et al.* [1934]. This possibility is supported by the observation, in this investigation, that only a part of the α - and β -glycerophosphatase activities is lost, whilst nearly all of the capacity for magnesium activation is lost, if unhaemolysed erythrocytes are allowed to stand for some time.

It is unlikely that this second enzymic factor is the acid organ phosphatase, unless it can be shown that the presence of erythrocytes, in the organ preparations they used, caused some of the phenomena observed by Davies [1934] and by Bamann and Riedel [1934]. Thus, at p_H 6.0 with 0.02 *M* $MgCl_2$, both the organ and the erythrocyte enzymes should be activated, and the sum of the increases should produce an apparent activation of the erythrocyte phosphatase greater than that observed in normal erythrocytes under these conditions. Such was not found. Furthermore, if the yeast enzyme which prefers the β -ester is analogous to the organ enzyme, one would expect to find that the progressive inhibition which Albers and Albers [1934, 2] found in this acid phosphoesterase with increasing magnesium chloride concentrations would be reflected in cancer erythrocytes. That is, the apparent activity should be decreased, because this inhibition should detract from the effect of magnesium on the true erythrocyte phosphatase. This decrease with high concentrations of magnesium chloride was found, but with the α - as well as the β -glycerophosphatase, and the organ enzyme is not supposed to be responsible for much of the attack on the α -isomeride. Another argument against the presence of the organ enzyme may be offered. A normal ratio of rate of hydrolysis of α -glycerophosphate to that of β -glycerophosphate, when a β -ester-preferring enzyme would not be expected

to be present in increased amount, is accompanied by a decreased capacity for magnesium activation in cancer erythrocytes.

If this enzymic factor is a natural activator or a phosphatase accompanied by a natural activator, there are no certain indications as to whether it is the same for α - as for β -glycerophosphatase. There is, however, a distinct possibility that it is not the same in view of the stability of the β -glycerophosphatase in the normal individual, whilst the α -glycerophosphatase shows not only considerable variation from individual to individual but also susceptibility to climatic conditions.

It is not possible on the basis of my experimental evidence to identify the source of the abnormal behaviour of cancer erythrocytes with regard to their glycerophosphatase, particularly with regard to their β -glycerophosphatase, activities. Nevertheless, it seems more likely that this source is to be found in abnormal conditions of activation, rather than in the admixture of the organ enzyme. Of interest should be the fact that whilst α -glycerophosphatase activity seems to be a rather unstable manifestation, the β -glycerophosphatase is constant and characteristic of the normal individual over a long period. Alterations in the β -glycerophosphatase activity, then, ought to be significant, especially since other substrates of physiological importance may also be attacked in an abnormal manner.

SUMMARY.

Normal and cancerous human erythrocytes hydrolyse α -glycerophosphate optimally at substrate p_H 5.6, usually at a rate which is about six times as great as that for the β -ester. The increase in α -glycerophosphatase activity with magnesium chloride is similar to, but not so great in proportion as, that in β -glycerophosphatase activity. The relative effect of magnesium chloride on both glycerophosphatases appears to be less in cancerous erythrocyte haemolysates than in normal erythrocyte haemolysates. Indications have been found that a part of the erythrocyte glycerophosphatase activity is independent of the presence of magnesium ions. The erythrocyte β -glycerophosphatase system seems to be much more stable than the α -glycerophosphatase system in the normal individual. It appears that it is the β -glycerophosphatase, and not the α -glycerophosphatase, system which has a tendency to become altered in cancer erythrocytes. A possible explanation for these phenomena is suggested.

Acknowledgment is made to the division of pathology of the laboratories of the Philadelphia General Hospital for its very helpful co-operation.

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CLIX. ON THE DEAMINATING ENZYME OF FLESH-FLY LARVAE.

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INTRODUCTION.

THE production of ammonia as their main nitrogenous end-product by flesh-fly larvae is now well known. The mechanism of its production however is still obscure [Messer and McLellan, 1935]. Hobson [1931] for instance was unable to find any evidence of deamination of glycine at p_H 6.8 by aqueous extracts of *Lucilia sericata* larvae. One of us [Brown, 1935] carried out the same experiment using larvae of *Wohlfahrtia vigil* on both glycine and tyrosine and varying the p_H from 4.0 to 8.0 with entirely negative results.

Some hope for the elucidation of this problem lay in an observation of Weinland [1908], who found that in his peptone brei of *Calliphora* tissue the reaction became definitely alkaline to litmus presumably owing to the production of ammonia. In the present communication the presence of a deaminating enzyme has been established and some of its properties and conditions of action have been determined.

ANALYTICAL METHODS.

Ammonia was determined by permutit adsorption and nesslerisation [Peters and Van Slyke, 1932]. In analysing solutions containing protein or its breakdown products the colour on nesslerisation was unsatisfactory owing to a straw-yellow tinge which developed; this could be obviated by repeated washings of the permutit after ammonia adsorption or by substitution of permutit adsorption by aeration into 0.1N HCl.

Amino-nitrogen was estimated by alcoholic titration [Willstätter and Waldschmidt-Leitz, 1921] using N/50 alcoholic NaOH with cresolphthalein as indicator.

Protein-nitrogen was measured by precipitating the protein with trichloroacetic acid and determining the nitrogen in the washed precipitate using the technique described by Farber and Wynne [1935].

Uric acid was determined by the method of Benedict and Franke [v. Peters and Van Slyke, 1932].

The organisms used in this investigation were *Lucilia sericata* Mg. and *Calliphora erythrocephala* Mg., both flesh-flies of the same ecological habit. Notes on their laboratory breeding are published elsewhere [Brown, 1936, 2].

Preliminary investigations on brei.

Brei was prepared in the following manner. Large numbers of washed larvae were ground in a mortar for 20 min. Of the resultant sticky mass portions were removed in a horn spoon, weighed and transferred to another mortar. They were then each ground with one-half their weight of the appropriate solution of the substrate and enough water (measured) was added to make the resultant brei thin enough to pour into a weighed centrifuge-tube which was then weighed

with its contents. These mixtures were incubated under toluene for 24 hours at 30°. They behaved exactly as described by Weinland [1908]. At the end of the digestion period water was added to 40 ml., the contents were well stirred and then centrifuged and aliquot amounts of the supernatant liquid were analysed for ammonia. Controls without substrate were also set up and the small value of their autolytic ammonia production, calculated on the same unit weight of original tissue, was subtracted from the experimental values.

It was found that ammonia was produced in large quantities from Witte peptone. Mere alkalinity (p_H 7.5-9.5) did not give rise to any production of ammonia from peptone under these conditions.

It was necessary to establish the variations in the deaminating activity throughout the life-cycle of the organism. Using brei of *Lucilia* on Witte peptone the results in Table I were obtained.

Table I. *Variation in deaminating activity of tissue brei during the life-cycle of Lucilia.*

		NH ₃ per g. tissue mg.	Weight per 100 larvae g.	NH ₃ per 100 larvae mg.
Larvae	Half-gorged	8.66	5.26	45.0
		8.47	—	—
	Fully gorged	11.11	4.80	53.3
	25% with gut evacuated	10.74	4.49	46.1
		9.69	—	—
	2% pupated	7.63	3.53	26.9
Pupae	1-3 days after pupation	1.32	4.74*	6.5
Adults	1-3 days after emergence	0.00	1.60	0.0

* Batch of exceptionally large-sized pupae.

The deamination therefore is at a maximum during the period of the most intensive digestion of protein by the larvae. It drops rapidly, being low in pupae and disappearing finally in the adult. It seems best to take larvae just before

Table II. *Deamination of various nitrogenous compounds by tissue brei of Lucilia.*

Substrate	NH ₃ in mg. per g. of tissue				
	Total production		Autolysis control II	In substrate III	Actual production I minus II plus III
	Values	Average I			
Witte peptone	13.23	13.14	4.50	0.07	8.57
	13.04				
Caseinogen, B.D.H.	16.07	14.94	4.50	0.07	10.27
	13.82				
Ovalbumin, Merck	2.75	2.89	2.28	0.30	0.31
	3.03				
Bactopeptone, Difco	3.68	3.84	2.28	0.15	1.41
	4.00				
Glycine	3.84	3.84	3.85	0.00	-0.01

Also no NH₃ production from these other amino-acids:

α -Alanine
 β -Alanine
Leucine
isoLeucine

Aspartic acid
Glutamic acid
Arginine

Phenylalanine
Tyrosine
Histidine

pupation, when (a) their gut is empty of contents which might interfere with the experiments, and (b) they can be easily obtained in a clean dry condition from the sawdust into which the larvae crawl on leaving the meat; although their deaminating power is not at its height, it is quite enough to work with.

The deamination of various nitrogenous compounds by larval brei was next investigated; the results are summarised in Table II.

The proteins and their more complex derivatives furnish the best substrates. Bactopeptone is composed of lower breakdown products than Witte peptone. Ovalbumin is exceptional, presumably owing to its resistance to proteolysis [Farber and Wynne, 1935]; moreover, it is a poor substrate for growth and ammonia production for these organisms *in vivo* [Brown, 1936, 1]. All the amino-acids studied are resistant to deamination.

Active preparations in powder form.

Powders were prepared in large quantities in the following manner. Up to 100 g. of larvae were sifted out from sawdust, washed under water for 1 hour, dried on filter-paper and well macerated in a large mortar. The tissue was then poured into acetone (ether, light petroleum or alcohol partially inactivated the enzyme) and the first extract was decanted through cheese-cloth. By kneading the material in the cloth with a stirring rod in frequent changes of acetone all the tissue except the skins came out in a coarse suspension which was poured through a filter. It dried on the filter-paper within an hour and could be readily pulverised; after passing through a 60-mesh wire screen it gave a powder of the texture of Witte peptone.

The activity of such powders was tested and found to equal that of the dried tissue; moreover, preparations of *Calliphora* and *Lucilia* showed the same order of activity. These experiments were allowed to continue for 4 days by decanting off the supernatant liquid (for ammonia determination) and leaving the centrifugate; they were stopped when the boiled controls began to show some ammonia production from bacterial contamination. At this point nearly 40% of the peptone-nitrogen had been converted into ammonia. Fig. 1 illustrates the results. These powders could be kept in the refrigerator without perceptible loss of activity over a period of weeks.

The substitution of extract for brei.

The solubility in water and in 50% glycerol of the enzyme system was next investigated. Larval powders were triturated in the solvent and centrifuged and the supernatant and centrifugate were tested. Both proteinolytic and deaminating activities were investigated; the results are shown in Table III.

Three types of active preparations were used:

- (1) The dry larval powder triturated in water.
- (2) A 50% glycerol extract of the larval powder.
- (3) A 50% glycerol extract of freshly ground larvae.

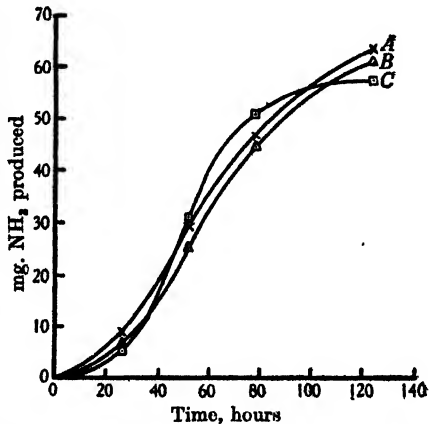


Fig. 1. Deaminating activity of dry preparations from brei. A, dried tissue, *Lucilia*; B, acetone powder, *Calliphora*; C, acetone powder, *Lucilia*.

Table III. *Solubilities of deaminase and proteinase of larval powders in water and 50% glycerol.*

	Water				50% glycerol			
	Supernatant		Centrifugate		Supernatant		Centrifugate	
	Protein-N	NH ₃ -N	Protein-N	NH ₃ -N	Protein-N	NH ₃ -N	Protein-N	NH ₃ -N
Initial, mg.	20.1	0.77	25.3	0.29	27.0	0.79	24.6	0.30
24 hours' digestion, mg.	6.5	2.50	3.9	3.47	9.5	4.27	7.8	3.20
Change in mg.	13.6	1.73	21.4	3.18	17.5	3.48	16.8	2.90
% of total activity	39	35	61	65	51	56	49	44

The digestion of caseinogen by the larval enzyme system.

Duplicate digests were set up of the larval powder with 1% caseinogen (B.D.H., later Kahlbaum's preparation according to Hammarsten was used) solution of an initial p_H of 8.6 in Erlenmeyer flasks at 37.5° with shaking under toluene. The p_H dropped to 7.0 during digestion. Residual protein-N, free carboxyl groups and ammonia were determined at appropriate intervals. The progress of two different experiments (with different enzyme preparations) is shown in Fig. 2.

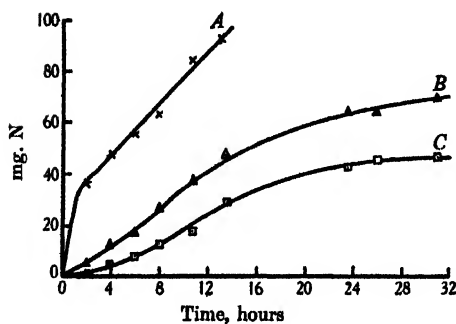


Fig. 2A.

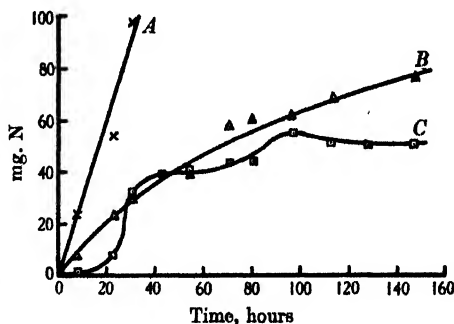


Fig. 2B.

Fig. 2. The breakdown of caseinogen by larval preparations. A, acetone powder 2 (*Calliphora*); B, acetone powder 4 (*Calliphora*).

A, protein-N, decrease; B, amino-N; C, ammonia-N, scale magnified $\times 5$.

It is evident that the protein decreases and free amino-groups increase in a fashion characteristic of "tryptic" digestion [Farber and Wynne, 1935]. The production of ammonia however occurring in considerable amounts after a short lag period is quite different from that in a tryptic digest.

In order to show the difference between digestion by trypsin and by the larval preparations a "switch" experiment was devised. A tryptic digest of caseinogen was set up; at three different points in the digestion a portion was removed, boiled and submitted to the larval enzyme. The addition of the larval enzyme immediately accelerated the production of ammonia. Data are presented in Fig. 3.

Three conclusions may be drawn:

(1) The larval enzyme produces ammonia much more quickly and in far greater amounts than does trypsin.

(2) The stimulating effect of the addition of the larval enzyme is also shown in the accelerated production of free amino-groups.

(3) The best substrates for the larval deaminase are those products which occur in the middle of the tryptic digestion, when the fragments of protein breakdown are still large.

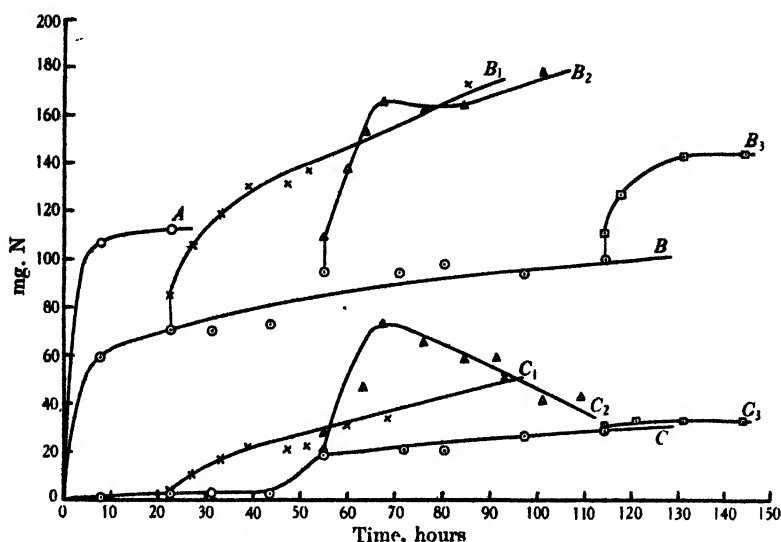


Fig. 3. "Switch" experiment. A, protein-; B, amino-; C, ammonia-nitrogen. Continuous lines from origin represent tryptic digestion. Samples removed at 23, 55 and 114 hours, boiled and submitted to larval enzyme.

The substrate for the larval deaminase.

The last experiment was repeated in greater detail: frequent samples of a tryptic digest were taken, boiled, made up to their original volumes and submitted to the action of the larval enzyme (in the form of a 50 % glycerol extract of fresh tissue) for 24 hours at 37.5° under toluene; the p_H was adjusted to 7.6, the optimum of the deaminase (see below). Typical results are given in Table IV. The first column represents the length of time for which the sample had been submitted to tryptic digestion and the second column the amount of ammonia that was produced from it by the larval enzyme.

Table IV. *Variation in deamination of tryptic digest of caseinogen by a glycerol extract of Calliphora.*

Time hours	NH ₃ -N mg.	Time hours	NH ₃ -N mg.	Time hours	NH ₃ -N mg.
0.0	0.77	13.2	0.86	152.0	0.58
1.2	0.93	22.5	0.90	Erepsin added	
3.6	0.89	25.8	1.03	152.5	0.46
4.7	0.95	29.0	0.91	157.5	0.53
6.7	0.99	35.0	0.73	166.5	0.66
8.5	1.00	37.8	0.91	193.0	0.30
10.2	0.99	48.0	0.59	286.0	0.36
12.0	0.95	77.2	0.41	359.0	0.27

Erepsin, in the form of a 50 % glycerol extract of fresh hog intestinal mucosa, was added to complete the digestion. Although the individual values are erratic, the following conclusions may be drawn tentatively:

(1) The original protein is not as good a substrate as its higher breakdown products.

(2) The most effective substrate is present over a broad range of tryptic digestion.

(3) Deamination falls off as the digest approaches the free amino-acid stage.

(4) Addition of the intestinal extract has a short stimulating effect on deamination, presumably due to deamination of the breakdown products of the protein contained in it.

The question of the best substrate was then examined from another angle. Although amino-acids were resistant to deamination, it seemed possible that mixtures of them would be susceptible or that there might be some uninvestigated susceptible amino-acids. The mono-amino-acid fractions therefore from a tryptic digest of caseinogen, from an acid digest of caseinogen and from an acid digest of gelatin were submitted to the larval enzyme and found in no case to be deaminated.

Samples of two dipeptides (glycylglycine, leucylglycine) and of a tripeptide¹ (leucylglycylglycine) were next tested as substrates. In each case there was considerable hydrolytic activity but no deamination: see Table V.

Table V. *Behaviour of larval enzymes towards simple peptides.*

Substrate	M/10 glycylglycine		M/40 leucylglycine		M/50 leucylglycylglycine	
	Amino-N	NH ₃ -N	Amino-N	NH ₃ -N	Amino-N	NH ₃ -N
Initial, mg.	10.56	0.256	7.35	0.139	5.86	0.139
24 hours' digestion, mg.	17.76	0.264	14.70	0.132	11.72	0.126
Produced, mg.	7.20	0.008	7.35	-0.007	5.86	-0.013

Since these organisms are strongly uricolytic [Truszkowski and Chajkinówna, 1935] it was thought possible that the ammonia might be produced from the breakdown of uric acid. Digests were therefore made up of 0.1 % uric acid in a Li₂CO₃ solution buffered at *p*_H 7.6 and submitted to the action of the larval powder and of a 50 % glycerol extract of fresh larvae, respectively. Samples were taken after 24 and 48 hours, controls with the enzyme preparations alone being run concurrently. The results are presented in Table VI.

Table VI. *Relation of uricolysis to production of ammonia by larval preparations.*

Hours	Powder			Glycerol extract		
	Uric acid Digest	NH ₃ -N Digest	NH ₃ -N Control	Uric acid Digest	NH ₃ -N Digest	NH ₃ -N Control
	mg. per 90 ml.			mg. per 90 ml.		
0	40.0	—	0.35	37.8	—	0.67
24	28.0	1.25	1.18	29.2	1.09	1.04
48	17.9	1.68	1.32	18.4	1.09	1.08

The results indicate that the enzyme preparations are strongly uricolytic, but that an inconsiderable amount of ammonia is produced as a result of this process; this is in keeping with the suggestion of Robinson [1935] that the allantoin present in the excreta of these organisms is the product of uricolysis.

The possible presence of a "deamidase" in the enzyme preparations was next

¹ The authors wish to express their indebtedness and thanks to Dr Max Bergmann of the Rockefeller Institute for his very great generosity and courtesy in supplying samples of the peptides.

tested using 0.05 % asparagine as substrate at p_H 7.6. There was a slight production of ammonia; its small significance is indicated by the following data for digests of 10 ml. substrate-buffer and 2 ml. 50 % glycerol preparation.

	mg.
NH_3 -N produced by action of larval enzyme	0.172
Total amide-N in substrate	5.300
NH_3 -N produced by same enzyme preparation on tryptic digest of caseinogen	1.028

It had been hoped that the deaminase could be activated, *e.g.* by protein breakdown products. A long-term tryptic digest of caseinogen and a similar larval digest of caseinogen were dialysed for 2 days; the dialysate and non-dialysed residue in both cases were added in small quantities to a digest of 0.1 % glycine with a 50 % glycerol extract of fresh larvae. Controls without the addition of "activator" and also without glycine but with "activator" were run concurrently. The results are presented in Table VII.

Table VII. *The effect of addition of various fractions on the production of ammonia from glycine by larval preparations.*

All values: mg. NH_3 -N per 12 ml. digest.

"Activator"	Experimental with glycine I	Control without glycine II	"Activator" as substrate I minus III	"Activation" I minus II
Caseinogen digest dialysate	0.262	0.254	0.038	0.008
Caseinogen digest residue	0.282	0.282	0.066	0.000
Larval digest dialysate	0.231	0.230	0.045	0.001
Larval digest residue	0.242	0.242	0.026	0.000
Water, III	0.186	—	—	—

The data clearly show that there is no indication of activation. Incidentally, it may be seen that the non-dialysed residues may act slightly as substrates and the dialysates to a lesser degree.

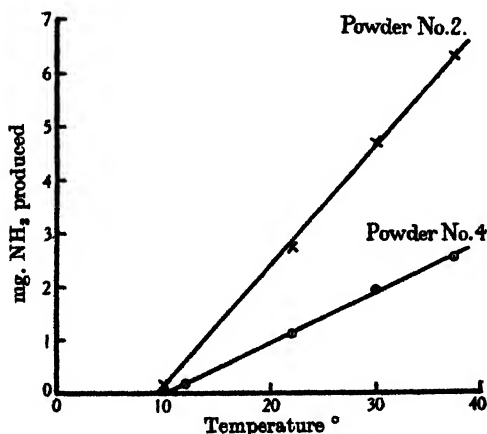


Fig. 4. Effect of temperature on deaminating activity.

Conditions regulating the activity of the enzyme.

The effect of temperature.

The activities of two different powder preparations of the deaminase at various temperatures are shown in Fig. 4.

The relationship between temperature and deaminase activity is linear over the temperature range investigated; temperature coefficients are as follows:

	Powder No. 2	Powder No. 4
10-20°	1.50	—
20-30°	1.94	2.04
30-40°	1.46	1.49

The effect of hydrogen ion concentration.

An incomplete study of the effect of p_H on the deaminase activity indicates that the optimum is about p_H 7.6. Fig. 5 shows the results obtained for two different enzyme preparations.

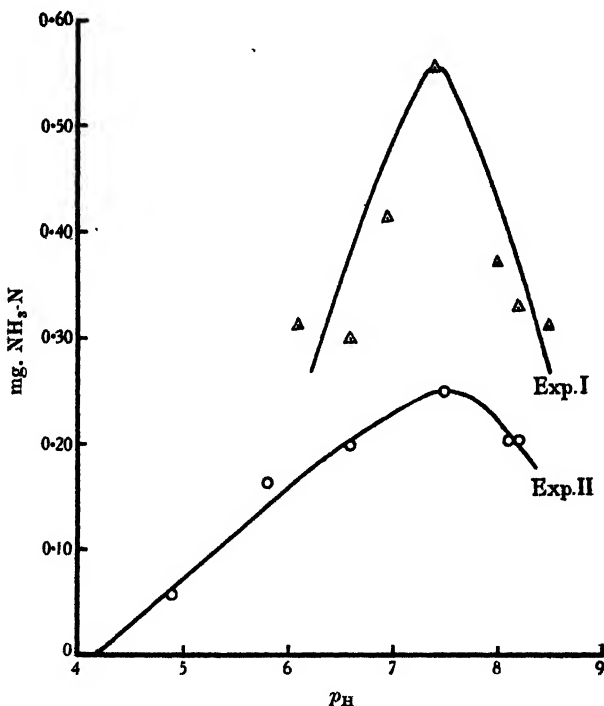


Fig. 5. Effect of p_H on deaminase activity.

Influence of oxygen.

For a long time it had been suspected that this deamination was an anaerobic process as suggested by Weinland's observations on brei. The first experiment in which digestion was carried out in presence of different gases is summarised in Table VIII a. The substitution of oxygen for air did not affect the deaminase activity whereas the presence of nitrogen accelerated the ammonia production. The low value under CO_2 is explicable by the depressing effect of the gas on the p_H .

In the second experiment digests in air were compared with those *in vacuo* using Thunberg tubes; the results are given in Table VIII b. Both sets of data show that the deaminase activity is greater under anaerobic conditions.

Table VIII. *Effect of gases on ammonia production from caseinogen by larval preparations.*

<i>a</i>			
Gaseous medium	Total production of $\text{NH}_3\text{-N}$ per g. powder	Actual production of $\text{NH}_3\text{-N}$ per g. powder	Final p_{H}
CO_2	6.08	4.32	5.9
O_2	8.09	6.33	6.3
N_2	9.48	7.72	6.5
Air	8.34	6.58	6.6
Control	1.76	—	—

<i>b</i>			
Conditions	Total production of $\text{NH}_3\text{-N}$ per 5 ml. digest	Actual production of $\text{NH}_3\text{-N}$ per 5 ml. digest	Average
Aerobic	0.638	0.395	0.391
	0.630	0.387	
Anaerobic	0.862	0.619	0.613
	0.850	0.607	
Control	0.243	—	—

Is this deamination oxidative or hydrolytic?

The fact that deamination proceeds better under anaerobic conditions is striking. It may be argued that this is an oxidative process in which oxygen is replaced as a hydrogen acceptor by other substances. Yet it seems unlikely that glycerol extracts of larval powders would be rich in such substances and moreover the process operates better in absence of oxygen; nor is the oxidative deamination in the mammalian kidney stimulated by addition of hydrogen acceptors [Krebs, 1933]. The enzyme itself cannot be destroyed by oxygen since the activity of preparations kept for long periods in air is unimpaired.

Moreover, α -hydroxy-acids have been detected in the deaminated residues. Difficulties were encountered in attempting to estimate hydroxyl groups by benzoyl chloride precipitation or by acetylation since these reactions are also given by amino-groups. Recourse was therefore had to methods of determination of lactic acid, which estimate also in lesser degree other α -hydroxy-acids. Residues in digests of the larval enzyme on caseinogen contained appreciable quantities of α -hydroxy-acids as indicated by the phenol-ferric chloride (Uffelmann's) test and by quantitative estimation with the method of Friedemann *et al.* [1927]. A digest was set up with gelatin, which has a relatively high alanine content, and the production of ammonia and "lactic acid" was followed. α -Hydroxy-acids were found to be produced at a rate roughly proportional to the ammonia production; however, in this substrate, the amount of digestion was too small to be of any great quantitative significance.

It is generally assumed that a deamination which is unimpaired by the absence of oxygen and which leaves α -hydroxy-acids in the deaminated residue is hydrolytic. But further work on the mechanism of deamination is indicated.

Separation of proteolytic and deaminating activities.

The enzyme preparations used are both proteolytic and deaminating; experiments hitherto conducted (*v.*, *e.g.*, Fig. 2 and Table III) gave no indication of their separate identities. In order to show that this ammonia production

involves something more than a special modification of proteolysis the two enzymes must be separated. Three types of experiment were devised with this end in view.

In the first a glycerol extract was precipitated with alcohol, but the results were not conclusive.

Experiments were then carried out in an attempt to differentiate between the two types of enzyme by varying the p_H . The optimum p_H for ammonia production is 7.6 (see above); however, the proteolytic activity at this p_H is not at its optimum. The p_H curves of proteolysis show two peaks, one in the neighbourhood of p_H 4.5 and the other at p_H 8.3. This suggests the existence of a tissue proteinase of the cathepsin type and of a proteinase of the trypsin type.

The third experiment was suggested by two observations of other workers; the one made by Hobson [1932] that excreta of *Lucilia* are proteolytic and the other made by Weinland [1907] that the excreta of *Calliphora* do not produce ammonia. A large number of *Lucilia* larvae were taken at the point of their maximum ammonia excretion [v. Brown, 1936, 1] and dropped into 100 ml. of distilled water; in this way a "washing" containing some of the excretory principles was obtained. The washed larvae were then placed on a sintered glass filter overnight, water being slowly added; in this way a solution of excreta was collected. The two solutions, "washings" and "excreta", were incubated at p_H 7.6 with caseinogen and with a tryptic digest of caseinogen, each digest being finally tested both for proteolysis and ammonia production. The results are shown in Table IX.

Table IX. *Proteolytic and deaminating activities of excreta of Lucilia.*

Digest	Substrate	"Enzyme"	NH ₃ -N		Protein-N	
			Initial	Final	Initial	Final
1	Caseinogen	Washings	0.04	0.04	14.1	1.7
1 A			—	0.04	13.8	1.6
2		Excreta	0.05	0.07	12.0	0.0
2 A			—	0.07	12.1	0.0
3	Tryptic digest of caseinogen	Washings	0.10	0.17	—	—
3 A			—	0.18	—	—
4		Excreta	0.13	0.17	—	—
4 A			—	0.17	—	—

The excreta are strongly proteolytic, but during this proteolysis virtually no ammonia is produced. This experiment thus demonstrates the separate identity of the proteinase and the deaminase and suggests that the latter, since it does not pass out from the digestive tract, is an intracellular enzyme. This is in accordance with the findings of Weinland [1908].

DISCUSSION.

The evidence now obtained agrees with the results of other workers and it seems permissible to attempt a tentative sketch of the protein metabolism in these organisms which are purely protein feeders. Ingested protein is broken down initially by proteinase thereby becoming a substrate for deamination which may take place in the cells of the posterior segment of the mid-intestine. The ammonia so produced may be excreted into the digestive tract without entering into the circulatory fluid where it would be injurious in such high concentrations; the deaminated products, probably α -hydroxy-fatty acids, diffuse into

the haemolymph and are used chiefly for fuel or may be synthesised into fat *in situ* [Weinland, 1908]. The products of proteolysis that escape deamination by proceeding too far towards the amino-acid stage may be absorbed into the blood and be used in the endogenous metabolism; they are finally excreted as allantoin [Robinson, 1935] which may be derived from uric acid [Brown, 1936, 1] by the action of uricase [Truszkowski and Chajkinówna, 1935].

The deaminase also possesses peculiarities of exceptional interest. In the first place, it is water-soluble; the only other water-soluble deaminase, as far as the authors are aware, is a bacterial aspartase [Virtanen and Tarnanen, 1931]. Secondly, it is entirely without action upon amino-acids and simple peptides, but is active on higher protein breakdown products; this appears to be unique. The only other instance in any way similar is that of an enzyme obtained from rose petals [Grassmann and Bayerle, 1934] which deaminates di- and tri-peptides more readily than amino-acids. This deaminase, however, required oxygen for its operation.

The third peculiarity of the deaminase of flesh-fly larvae is that its optimum activity is under anaerobic conditions. Examples of anaerobic deaminases are rare; kidney histidase, aspartase and the so-called "spontaneous" deamination of excised organs and tumours may be mentioned, but their function appears to be quite specialised; perhaps the same may be said of the interesting enzyme present in kidney, dehydropeptidase [Bergmann and Schleich, 1931], whose mode of operation is apparently hydrolytic. The only really parallel instance, *i.e.* involving the deamination of protein breakdown products anaerobically, with which we are familiar, is the production of α -hydroxy-isocaproic acid from leucine by *Clostridium acetobutylicum* [Schmidt *et al.*, 1924].

SUMMARY.

The deaminase of flesh-fly larvae has been extracted and studied.

1. The enzyme operates better under anaerobic conditions.
2. The optimum p_H for its operation is 7.6.
3. The relation of its activity with temperature is linear over the range 10–37.5°.
4. The enzyme is water-soluble.
5. It is an intracellular enzyme.
6. The substrates are not simple amino-acids, dipeptides or tripeptides but the higher breakdown products of proteins.

Preparations from these organisms were found to contain also proteinase, polypeptidase, dipeptidase, uricase and very slight deamidase activities.

The authors wish to express their gratitude to Prof. Hardolph Wasteneys and to Dr A. M. Wynne for unfailing help and advice, to Mr Donald Robertson for valuable assistance in rearing the insects and to Miss Jean P. Griffiths for performing the lactic acid determinations.

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CLX. THE ESTIMATION OF REDUCED ASCORBIC ACID IN BLOOD SERUM AND PLASMA.

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THE use of 2:6-dichlorophenolindophenol for the estimation of ascorbic acid was introduced by Tillmans *et al.* [1932]. The specificity of the reaction under suitably controlled conditions has been well established by Harris and Ray [1933], Birch *et al.* [1933] and Johnson and Zilva [1934]. These authors have applied the method variously to the determination of ascorbic acid in urine, tissues and food materials.

The purpose of the present communication is to describe a method for the estimation of small amounts of ascorbic acid in blood. The presence of large amounts of protein in blood serum and plasma renders the direct titration by dichlorophenolindophenol (Tillmans's reagent) impossible. Several methods have been employed for the removal of protein from serum or plasma before titration with this reagent [Mirsky *et al.*, 1935; Roe, 1935; Gabbe, 1934; Emmerie and Van Eekelen, 1934; Farmer and Abt, 1935]. The most widely used method has been precipitation by trichloroacetic acid. However, this reagent is not satisfactory for this purpose because it slowly decolorises the dichlorophenolindophenol indicator, thus making a sharp end-point with the ascorbic acid titration impossible. Farmer and Abt [1935], seeking to avoid the unsatisfactory end-point occasioned by the presence of trichloroacetic acid, resorted to sodium tungstate-sulphuric acid as a protein precipitant, finding that it did not interfere in the titration with Tillmans's reagent. During the past year, working independently, we have employed tungstic acid precipitation in the routine determination of the ascorbic acid content of the blood serum and plasma.

PROCEDURE.

The procedure should be commenced as soon as possible after the blood is drawn to diminish any tendency for cellular breakdown or oxidation of the vitamin. A 2 ml. sample of blood serum (or plasma) is pipetted into each of two 50 ml. centrifuge-tubes to provide for duplicates. 14 ml. of water and 2 ml. of 5 % sodium tungstate are added to each tube. The protein is then precipitated by the addition to each tube of 2 ml. of $N/3$ H_2SO_4 . The contents of the centrifuge-tubes are well mixed and allowed to stand until precipitation is complete and are then centrifuged for 10 min. The supernatant liquid is decanted into Erlenmeyer flasks and acidified with 0.5 ml. of glacial acetic acid. The flasks containing the supernatant liquid from the successive washings of each sample should be kept stoppered in a drawer in the dark until titrated.

The protein precipitate is redissolved in 2 ml. of 5% sodium tungstate solution by stirring and diluted with water. The amount of water added, together with that used in washing the stirring rod, approximates to 14 ml. The protein is then reprecipitated as before. This re-solution, precipitation and centrifuging is repeated four times; the supernatant liquid being added each time to the previous washings in the Erlenmeyer flasks.

The contents of each of these flasks are titrated with Tillmans's reagent prepared and standardised as given below. 1 ml. of this solution is equivalent to approximately 0.10 mg. of ascorbic acid. The titration is carried out rapidly to a standard end-point, the colour produced by 1 drop of Tillmans's reagent added to boiled distilled water of approximately the same volume as the unknowns, and containing 0.5 ml. of glacial acetic acid in an Erlenmeyer flask similar to that used for the unknown solutions. Whilst the end-point is not fugitive, more consistent results can be obtained by arbitrarily choosing as an end-point the number of ml. of Tillmans's reagent required to produce a colour matching the standard which will persist for 1 min. or more. The "blank" introduced by this method, amounting to not more than 0.005 mg. of ascorbic acid, is then deducted from the value obtained.

Reagents.

1. *Sodium tungstate*. C. P. Sterling Certified, 5% solution, freshly prepared weekly.
2. *Sulphuric acid*. *N*/3, freshly prepared weekly.
3. *Acetic acid*. C. P. "Baker's Analysed" glacial.
4. *Ascorbic acid*.¹ ("Cevitamic Acid" Merck) Standard solution 1 mg. per ml. (see directions below).
5. *2:6-Dichlorophenolindophenol* (La Motte). 0.2 mg. per ml. (see directions below).
6. *Iodine*. *N*/100 solution in potassium iodide. (Prepared according to Treadwell and Hall [1924].)
7. *Starch indicator*. Prepared according to the method of Treadwell and Hall [1924].

Preparation of standard solution. The standard solution is prepared by dissolving 100 mg. of pure ascorbic acid in 100 ml. of boiled, distilled water. Although the samples of ascorbic acid in the solid form were pure, it was found that the acid was quite rapidly oxidised in solution. The reduced ascorbic acid, remaining in the standard solution, was checked daily by determining its reducing power with *N*/100 iodine solution, using the method of Treadwell and Hall [1924]. The iodine titration values have been found to fall to about one-half in the course of 1 week. The standard ascorbic acid solution should be preserved in a brown glass stoppered bottle and stored in the ice-chest.

Preparation of Tillmans's reagent. 20 mg. of 2:6-dichlorophenolindophenol² are dissolved in 75 ml. of boiling distilled water, cooled, transferred to a 100 ml. volumetric flask and made up to the mark with distilled water. It is then checked daily against the standard ascorbic acid solution. The reagent should be replaced within 2 weeks in order to maintain a sharp end-point. Both Tillmans's reagent and the standard ascorbic acid solution should be protected from light in dark

¹ To Prof. Szent-Györgyi and to the Merck Company, Rahway, N.J., we gratefully acknowledge the gifts of ascorbic acid used in these experiments.

² Sometimes a reddish impurity is found in the commercial product which must be removed by extraction with ether. Alternatively the dye may be prepared quite simply in the laboratory by the method of Cohen *et al.* [1924] as suggested by Bessey and King [1933].

bottles and stored in the ice-box. In all titrations involving the use of Tillmans's reagent the solutions to be titrated are brought to the acid side of Congo red (p_H 4·7) by addition of glacial acetic acid. In the method described 0·5 ml. of glacial acetic acid was usually sufficient for this purpose.

Precipitant. Sodium tungstate-sulphuric acid was adopted as a protein precipitant after many trials of other substances. It was finally used in the method for two reasons: it avoided the fugitive end-point of trichloroacetic acid and the solubility of the protein precipitate in an excess of sodium tungstate afforded a ready way of washing the precipitate free from contained ascorbic acid.

Interfering agents. Under the experimental conditions outlined below the following substances were found not to interfere: phenols, uric acid, amino-acids, urea, creatinine, glucose. As has been reported previously, thiosulphate interferes [Harris and Ray, 1933].

EXPERIMENTAL.

A fairly definite percentage of ascorbic acid is left in the protein precipitate. Several experiments have shown that about 50 % may be recovered without washing, and that after each subsequent washing up to 30 % of the ascorbic acid remaining is occluded. After 4 washings, when small amounts of ascorbic acid are added to the untreated serum, between 89 and 111 % is recovered. Table I gives recoveries obtained from ascorbic acid added to serum or plasma in amounts ranging from 0·0151 to 0·272 mg.

Table I. *Recoveries of ascorbic acid added to blood serum.*

No.	Ascorbic acid present in 2 ml. blood serum mg.	Ascorbic acid added mg.	Total amount ascorbic acid found mg.	Ascorbic acid recovered mg.	Loss or gain mg.
1	0·0386	0·0151	0·0551	0·0165	+ 0·0018
2	0·0316	0·0159	0·0468	0·0154	- 0·0005
3	0·0489	0·0212	0·0696	0·0207	- 0·0005
4	0·043	0·0282	0·0705	0·0275	- 0·0007
5	0·0386	0·0302	0·071	0·0324	+ 0·0022
6	0·0489	0·032	0·079	0·0301	- 0·0019
7	0·045	0·0405	0·0855	0·039	- 0·0015
8	0·045	0·0405	0·0834	0·0384	- 0·0021
9	0·0386	0·0453	0·0875	0·0489	+ 0·0036
10	0·0489	0·0472	0·0960	0·0473	+ 0·0001
11	0·043	0·0564	0·0960	0·053	- 0·0034
12	0·0386	0·0604	0·0938	0·0552	- 0·0052
13	0·034	0·068	0·101	0·067	- 0·001
14	0·034	0·102	0·136	0·102	± 0·000
15	0·043	0·1128	0·145	0·102	- 0·010
16	0·034	0·136	0·167	0·133	- 0·003
17	0·037	0·154	0·174	0·137	- 0·017
18	0·034	0·204	0·244	0·210	+ 0·006
19	0·030	0·216	0·230	0·1995	- 0·016
20	0·034	0·272	0·319	0·285	+ 0·013

In the recently published method of Farmer and Abt [1935] the proteins are also precipitated by sodium tungstate-sulphuric acid. The determination is made on plasma. It has been found that the plasma and serum values agree very well provided that, in addition to the procedure outlined by Farmer and Abt, the proteins are reprecipitated at least three times and washed. Without this precaution, the values were found to be relatively low. The effect of washing and reprecipitation is shown in Table II.

Table II. *Effect of washing and reprecipitation of the protein filtrate on recovery of ascorbic acid from serum and plasma.*

Subject	mg. ascorbic acid per 100 ml. serum (1 washing)	mg. ascorbic acid per 100 ml. serum (4 washings)	mg. ascorbic acid per 100 ml. plasma (1 washing)	mg. ascorbic acid per 100 ml. plasma (4 washings)
Gr.	0.30	0.69	0.34	0.80
McC.	—	1.03	—	0.93
Wa.	—	—	0.18	0.32
Mc.	0.19	0.57	—	—
Sh.	—	0.60	—	0.69
Za.	—	0.87	—	0.79
Gr.	—	0.89	—	1.13
Fa.	—	1.35	—	1.31
Fa.	0.87	1.73	0.82	1.73
Mo.	1.73	2.59	1.59	2.41
Ma.	0.29	0.41	—	—
Gr.	0.70	1.33	—	—
Ch.	0.25	0.57	—	—

Applications. Studies on the output of ascorbic acid in the urine of normal individuals have shown wide variations dependent, to some degree at least, on the intake. The level of ascorbic acid in the blood of normal individuals and patients without infections likewise showed differences roughly parallel with the dietary intake of the vitamin. Table III illustrates values found in a few of a series of individuals representing, from the dietary point of view, a cross section

Table III. *Ascorbic acid content of normal individuals and control patients.*

Subject	Diagnosis	Ascorbic acid content of serum mg. ascorbic acid per 100 ml. serum
Fi.	Normal	2.24
Da.	"	2.03
Cu.	"	1.93
Ta.	"	1.90
Ow.	"	1.70
Fa.	Hypertension	1.69
McG.	Normal	1.63
Pa.	"	1.61
Na.	"	1.60
Fe.	"	1.59
Pl.	Spastic constipation	1.54
He.	Normal	1.50
Fa.	"	1.38
Lo.	Cirrhosis of liver	1.35
Ab.	Tacnia saginata	1.25
La.	Hypertension	1.23
Pa.	Anxiety neurosis	1.18
Fl.	Normal	1.04
Wo.	Diabetes mellitus	0.85

of normal individuals in the community. The group contained adults of both sexes and all ages comprising members of the staff and such hospital patients as were free from infections or clinical evidence of dietary deficiency. These determinations were all carried out in the months of July to October, inclusive, in Boston. The average value in 33 such individuals was 1.61 mg. per 100 ml. of serum of reduced ascorbic acid with extremes of 2.43 and 0.83 mg. respectively.

In 10 adult patients with clinical manifestations of scurvy the level of ascorbic acid in the blood was relative low, varying from 0.11 to 0.55 mg. per 100 ml. with an average of 0.245 mg. per 100 ml. (see Table IV).

Table IV. *Blood ascorbic acid in clinical scurvy.*

Case No.	Subject	mg. ascorbic acid per 100 ml.	Case No.	Subject	mg. ascorbic acid per 100 ml.
1	Sm.	0.11*	6	Ta.	0.20*
2	Bn.	0.11*	7	Ba.	0.26*
3	Co.	0.13*	8	O'H.	0.30*
4	Wa.	0.18*	9	Wi.	0.42
5	Jo.	0.19*	10	Re.	0.55
Average					0.245

* The experimental error, being 0.2 mg. per 100 ml., becomes relatively so great with the lower values that it is perhaps preferable to designate any value below 0.4 mg. per 100 ml. as a "trace" rather than to assign a quantitative figure to it.

The effects of the oral and intravenous administration of ascorbic acid on the blood level of this substance are shown in Figs. 1 and 2. The subjects were fasting throughout the experiment. Fig. 1 represents the observations in a normal young adult. The four determinations taken at hourly intervals in a

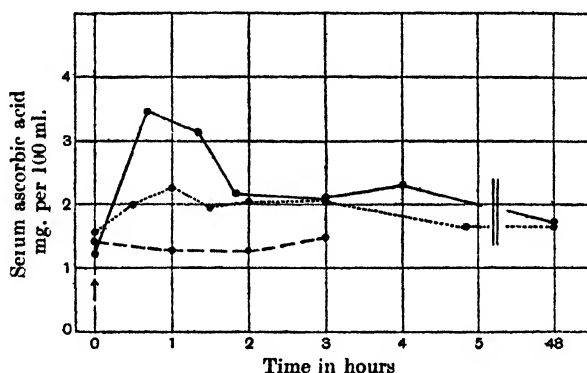


Fig. 1. Ascorbic acid of blood serum after administration of 1 g. ascorbic acid to a normal subject.
 ↑ Time of administration. — Intravenous. ---- Oral. . . . Fasting control.

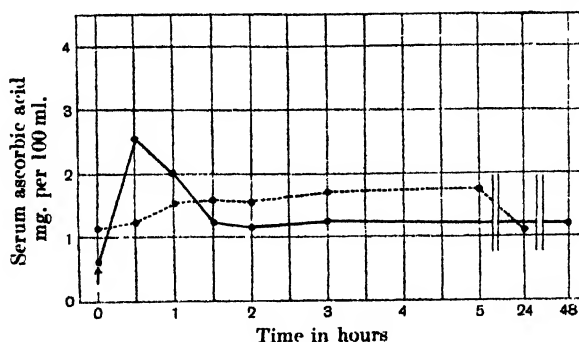


Fig. 2. Ascorbic acid of blood serum after administration of 1 g. ascorbic acid to a scurvy patient.
 ↑ Time of administration. ---- Oral. — Intravenous.

fasting condition were constant within experimental error and act as a control for the experiment in this subject. On oral administration of 1 g. of ascorbic acid, the serum level rose from a base line value of 1.57 mg. per 100 ml. to a peak of 2.29 mg. in 1 hour, returning in 5 hours to approximately the initial level. 1 g.

of the vitamin was given intravenously 3 weeks after the previous experiment, at which time the fasting blood serum level of ascorbic acid was 1.21 mg. per 100 ml. 30 min. after injection, the serum value was 3.49 mg. per 100 ml., from which it fell to 2.17 mg. per 100 ml. in 2 hours. At the end of 4 hours the serum value was 2.30 mg. per 100 ml., still significantly higher than the initial level.

Fig. 2 illustrates the findings in a case of scurvy. 1 g. of ascorbic acid was administered intravenously and the blood level of ascorbic acid was determined after 30 min., 1, 1½, 2, 3 and 48 hours. The blood serum level of ascorbic acid rose sharply from an initial level of 0.61 mg. per 100 ml. of serum in 30 min. to 2.58 mg. per 100 ml. of serum. In 1½ hours the value had fallen to 1.24 mg. per 100 ml. and was maintained at this level for at least 48 hours. The curve following oral administration was obtained 7 days later, the patient having been kept on a vitamin C-free diet. The blood level had fallen during this interval to 1.14 mg. per 100 ml. of serum. From this initial level the ascorbic acid content rose very gradually after oral administration of 1 g. of the vitamin in aqueous solution, reaching a level of 1.70 mg. per 100 ml. 3 hours after ingestion. This value was maintained for at least 2 more hours. 24 hours later the ascorbic acid content of the serum had fallen to approximately the initial level.

DISCUSSION.

The transient rise in the blood level of ascorbic acid following administration of the pure substance furnishes indirect confirmatory evidence of the specificity of the method itself. This observation and the finding of relatively low blood ascorbic acid levels in scurvy are in keeping with the suggestion of Farmer and Abt [1935] that the level of reduced ascorbic acid in the blood may be used as a measure of the "nutritional state" of the organism with respect to vitamin C.

The method is presented as a relatively simple and reliable procedure for the estimation of the level of reduced ascorbic acid in blood serum or plasma. As such, it offers a useful tool for the study of the effects of diet and disease upon the metabolism of vitamin C. A report of such studies will be the subject of another communication.

SUMMARY.

1. A method is described for the estimation of reduced ascorbic acid in 2 ml. samples of blood serum or plasma.
2. Recoveries of added ascorbic acid range between 89 and 111 %.
3. In 33 individuals, free from infection or scurvy, the average level of reduced ascorbic acid in the blood serum was 1.61 mg. per 100 ml., with extreme values of 0.83 and 2.43 mg. per 100 ml.
4. Of 10 individuals with scurvy, 8 showed values of reduced ascorbic acid in the blood serum below 0.4 mg. per 100 ml., which is the smallest amount which can be quantitatively determined by the method used. The remaining 2 showed values of 0.42 and 0.55 mg. per 100 ml. respectively.
5. Typical curves are shown illustrating the transient rise in the blood level of reduced ascorbic acid following oral and intravenous administration of the pure substance in a normal subject and in a case of scurvy.

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CLXI. CEREALS AND RICKETS.

VII. THE ROLE OF INORGANIC PHOSPHORUS IN CALCIFICATION ON CEREAL DIETS.¹

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THE possibility that a particular agent is responsible for the poor calcifying properties of cereal grains has been a focal point of interest to students of nutrition for some years. It has long been recognised that grains are a poor source of Ca, yet this deficiency has failed to provide an adequate explanation.

E. Mellanby [1921; 1922, 1, 2; 1924; 1925; 1926, 1, 2; 1930] observed that a high cereal diet invariably had a deleterious effect upon the calcification of bones and teeth in dogs and rats. Oatmeal appeared unique in producing a greater severity of rickets than other cereal grains. Mellanby [1926, 2] proposed the idea that cereals contain some distinct anticalcifying substance which he provisionally called a toxamin. This anticalcifying action was found by Green and Mellanby [1928] to be neutralised by simultaneous administration of vitamin D, or by exposure of the cereal to ultraviolet light, according to the findings of Steenbock [1924] and Steenbock and Black [1924].

M. Mellanby [1928; 1929] emphasised the anticalcifying effect of cereals on teeth. Consideration of the chemical analyses of the cereals revealed that those which had the worst effect on teeth frequently contained the most Ca and P. Furthermore, the Ca/P ratio was not found responsible.

Green and Mellanby [1928] showed that oatmeal could be boiled with water or heated in the dry state at 120° for 18 hours without correction of its anticalcifying properties. If, however, it were boiled with 1 % HCl for 1.5 hours and then neutralised, the product was found to have lost its anticalcifying effect.

Holst [1927] reported that oats contained a rickets-producing factor which could be extracted with 0.5 % HCl. Mirvish [1929; 1930] reported that when a dilute HCl extract of oats was injected into animals it produced a marked fall of blood Ca. Later Mirvish and Bosman [1929] suggested the identity of the blood Ca-reducing principle with the anticalcifying factor. Christiansen [1934] extracted oat flour with 0.5 % HCl and then dialysed the extract against distilled water. The dialysate when injected into rabbits produced a marked fall in serum Ca. However, similar effects on the serum Ca were obtained by injecting substances which had no anticalcifying properties.

Fine [1930] corroborated Mellanby's work with respect to the differences in the calcifying properties of cereals, but he ascribed the difference to variations in the vitamin D content. Harris and Bunker [1931] investigated the irregularity in the production of rickets on a rachitogenic diet and traced the difficulty to the yellow corn component in Ration 2965. They reported that the trouble could be

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corrected by storing the ground corn meal for a few months before using. In a later paper [1934], these workers emphasised the variations in the P content of samples of fresh yellow corn obtained from different localities and showed that the Ca/P ratio in various rachitogenic rations may vary widely. Holmes and Tripp [1933] and Davies [1934] also reported widely different Ca/P ratios on various samples of Ration 2965, due chiefly to differences in P content.

Steenbock *et al.* [1927] have been unable to demonstrate that rolled oats are especially rickets-producing. They [1930] found that the cereals ranked in decreasing order of their anticalcifying effect thus: corn, rolled oats, wheat, regardless of whether they were fed alone or supplemented with CaCO_3 and with H_3PO_4 . Of particular significance is their observation "that equalisation of the phosphorus content of the cereal rations did not make them equally effective in bone formation" and their suggestion that "phosphoric acid may not be equivalent in physiological properties to the organic phosphorus compounds in the cereals". More recent work by Templin and Steenbock [1933, 1] demonstrated that superior calcification was produced by immature yellow dent field maize as contrasted with the corresponding mature maize of the same variety and grown under identical conditions. In another paper by the same authors [1933, 2] the striking antirachitic effect of autolysed germinated maize was pointed out as compared with both mature maize and germinated maize, and attention was again directed to a consideration of the phosphorus component.

Bruce and Callow [1934] reported that "the apparent rachitogenic effect of cereals when compared with other material of the same phosphorus content is due to the fact that cereal phosphorus is not in an available form" and that "the differences between oatmeal, maize and white flour can be completely accounted for by differences in the total phosphorus content and in the proportion of inositolhexaphosphoric acid". Treatment of oatmeal with 1% HCl was found to destroy its anticalcifying effect very strikingly as the inositolhexaphosphoric acid was hydrolysed. McCance and Widdowson [1935] found, after feeding phytin to 3 adults and 1 child, that 20–60% of the phytin was excreted unchanged in the faeces.

Eddy *et al.* [1922] had reported earlier that when the inorganic P of the diets of rats was replaced by phytin, the rats were not protected against rickets although the food intake remained the same. Harris and Bunker [1935] recently studied the phytin content of different samples of mature corn, but reported no correlation between the phytin content of the corn and the severity of rickets produced by it.

EXPERIMENTAL.

The striking effect of immature yellow dent maize and of germinated, autolysed maize in promoting calcification when contrasted with the mature seeds suggested the advisability of studying these materials for their organic-inorganic P relations. The experiments of Templin and Steenbock [1933, 1, 2] were, therefore, repeated with this objective.

Mature yellow dent field maize on the cob obtained from the University Farm was dried with forced air circulation at a temperature of 42° for 36 hours, after which it was shelled and stored in a dry place. It was ground just before using. The manner of preparing the maize for germination and autolysis has been described in an earlier paper [1933, 2]. In the present work, "germinated maize" refers to that which has been germinated for a period of 96 hours, "germinated autolysed maize" to that which has been germinated for the same time and then autolysed for 10 days.

The method of assay employed in our experiments was unvaried. Six groups of rats between 3 and 4 weeks of age and weighing 50–60 g. with six rats in each group were used to test each of six rations. Litter-mate rats were uniformly distributed among the groups. Food consumption was equalised, the daily food intake of each rat being restricted to the amount taken by the rat with the lowest food consumption provided that it was not obviously abnormal. In addition to the modified Ration 2965 and distilled water, each rat received supplementary carotene. This was administered by dropper in oil¹ solution, each rat being given 5γ in one drop on alternate days. All the rations were finely ground, with the CaCO₃ thoroughly incorporated to prevent its settling out. All rations represented modifications of Ration 2965 in which the yellow maize was wholly or partially replaced by yellow maize of special selection or treatment. The rats were weighed weekly. After 5 weeks they were killed with ether, the costochondral junctions examined and the femora analysed for ash after alcohol extraction.

In Series I, germinated and germinated-autolysed maize was used to replace partially the mature maize component of Ration 2965. Preliminary experiments had shown that partial replacement avoided poor food consumption and provided better growth than complete replacement. As an added precaution, 2 % of yeast was included in all the rations.

Determinations of inorganic P and total P were made according to an adaptation of the Fiske and Subbarow method [1925]. Use of 0.8 % HCl for the extraction of inorganic P completely prevented the action of phytase. Determinations of phytin-P were made according to Harris and Mosher's [1934] modification of the Heubner-Stadler method [1914]. We were unable to avail ourselves of the recently reported modifications of this method by McCance and Widdowson [1935] and Young [1936], since their papers did not appear until after completion of our work.

The results of the experiments of Series I are presented in Table I. The importance of the inorganic P relations in these rations is clearly evident. Germinated autolysed maize showed an antirachitic superiority over mature maize

Table I. Series I. *Relation of inorganic P content to the rachitogenic properties of germinated and germinated autolysed maize.*

No.	Modification	Ration 2965 Maize component		Animals Each figure is average of 6 rats						
		In- organic P %	Total P %	In- organic % of total	Ca/I ¹ ratio	Daily food con- sumed				
						Gain in wt. g.	Femora g.	Ash g.	Ash %	
1	Mature maize (basal)	0.02	0.33	6	4/1	45	7.7	0.103	0.030	28.8
2	Germinated autolysed maize, 50 %	0.14	0.33	42	4/1	34	7.7	0.124	0.055	46.2
3	Germinated autolysed maize, 25 %	0.07	0.33	21	4/1	45	7.6	0.115	0.043	38.5
4	Germinated maize, 50 %	0.06	0.33	18	4/1	29	7.1	0.093	0.022	24.2

as well as germinated maize. Determinations of the phytin-P of these rations revealed an inverse relation between the phytin-P content and the antirachitic effectiveness. Bruce and Callow's conclusions are thus generally confirmed.

¹ A vegetable oil sold under the trade name of Wesson Oil by Wesson Oil and Snowdrift Sales Company, New York.

Series II was designed to determine the effects of adding varying amounts of H_3PO_4 to the basal diet. H_3PO_4 was added to the rachitogenic ration in sufficient quantities to establish various arbitrary Ca/P ratios ranging from 4/1 to 1/1. The work of Shohl *et al.* [1932] was used as a guide. They stressed the importance of the Ca/P ratio in the study of rickets, but likewise showed the necessity of employing a basal rachitogenic ration of low P content. Our Ration 2965 was shown to have a Ca content of 1.2% and a P content of 0.3%, giving a Ca/P ratio of 4/1. The modifications of this ratio together with the results obtained are presented in Table II.

Table II. Series II. *Effect of various additions of H_3PO_4 to Ration 2965 on its rachitogenic properties.*

No.	Modification	Ration 2965		Animals						
		Maize component		Each figure is average of 6 rats						
		In-organic P %	Total P %	In-organic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
7	Mature maize (basal)	0.02	0.33	6	4/1	35	6.9	0.088	0.026	29.9
8	Basal + H_3PO_4	0.06	0.37	16	3.5/1	36	7.0	0.098	0.031	31.2
9	Basal + H_3PO_4	0.12	0.43	28	3.1/1	39	6.9	0.105	0.037	35.4
10	Basal + H_3PO_4	0.20	0.51	39	2.6/1	39	7.1	0.120	0.053	43.8
11	Basal + H_3PO_4	0.32	0.63	51	2.1/1	43	7.1	0.151	0.073	48.2
12	Basal + H_3PO_4	0.92	1.23	75	1.1/1	35	6.3	0.139	0.069	48.7

The ash analyses in Series II present a well-defined illustration of the effect of varying the supplements of H_3PO_4 in a rachitogenic diet. The progressive improvement in calcification up to and including a Ca/P ratio of 2.1/1 runs exactly parallel with the rise in P content. The failure of improved calcification with a Ca/P ratio of 1.1/1 as compared with 2.1/1 becomes clear when we examine the Ca/P relation from a stoichiometrical viewpoint. The theoretical amounts of Ca and P required to produce $Ca_3(PO_4)_2$ are 2 parts of Ca to 1 of P. The critical Ca/P ratio appears to be between 2.6/1 and 2.1/1. In later work, therefore, it was attempted to confine Ca/P ratios to the zone where the greatest change in calcification took place, *viz.* between 3.1/1 and 2.6/1.

Since phytin is by far the principal source of P in cereal grains, Series III was set up primarily to compare the availability of this form of P with other forms. According to early workers, phytin is absorbed and the P is excreted as inorganic phosphorus in the urine. Scofone [1905], Giascosa [1905], Mendel and Underhill [1906], Cook [1909] reported that organic phosphorus compounds could be assimilated as such and that they were preferable to inorganic phosphate as a source of P for animals. Forbes and Keith [1914] reported no fundamental difference in nutritive values of phosphorus compounds and therefore no basis for a differentiation between their nutritive values.

Rogozinski [1910] showed that in dogs the unabsorbed phytin was present as such in the faeces, whereas in man it was hydrolysed by the bacteria in the large intestine. Rather [1918] found that after feeding large amounts of phytin in the natural state to pigs almost all the P was excreted in the form of orthophosphoric acid. He concluded that the pig has the power to hydrolyse phytin completely.

A few contemporary workers are inclined to believe that phytin is actually toxic to the animal organism [*cf.* Stockman, 1934]. Stockman and Johnston [1933] reported the production of symptoms of nervous degeneration in monkeys

by using a cereal extract presumably containing inositolhexaphosphoric acid. These observations have not been confirmed by Bruce and Callow [1934].

To determine the effect of phytin as such, we prepared phytin according to Boutwell's [1917] modification of the method of Clarke [1914]. From 15 pounds of wheat bran a net yield of 160 g. of phytin was obtained—a white amorphous powder, insoluble in water, but readily soluble in dilute mineral acids. It contained less than 0.01 % inorganic P, 14.0 % total P, and 1.8 % Ca.

In Series III phosphoric acid was added to the basal Ration 2965 in amounts necessary to establish Ca/P ratios of 2.9/1 and 2.3/1 respectively. Other rations were supplemented with phytin to provide equivalent amounts of P, and in one case Na glycerophosphate was added instead.

The results of the ash analyses in this series (Table III) reveal that phytin as a source of P was without significant value. Equivalent amounts of P given as

Table III. Series III. *Availability of phytin-P as compared with inorganic P.*

No.	Modification	Ration 2965		In-		In-		Ca/P		Animals				
		Maize component		organic		organic		ratio		Each figure is average of 6 rats				
		In-organic P %	Total P %	In-organic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %				
13	Mature maize (basal)	0.02	0.33	6	4/1	44	8.4	0.110	0.030	27.8				
14	Basal + H_3PO_4	0.15	0.46	33	2.9/1	50	8.5	0.128	0.046	36.2				
15	Basal + phytin	0.02	0.46	4	2.9/1	39	8.2	0.106	0.030	27.8				
16	Basal + H_3PO_4	0.26	0.57	45	2.3/1	43	8.5	0.139	0.062	44.5				
17	Basal + phytin	0.02	0.57	3	2.3/1	42	8.5	0.108	0.032	29.4				
18	Basal + Na glycerophosphate	0.02	0.57	3	2.3/1	46	8.5	0.167	0.088	52.8				

phosphoric acid, however, resulted in a pronounced improvement in calcification. Na glycerophosphate was still more beneficial.

The limitations in the general applicability of these results, however, must be recognised, because Ration 2965 is not a balanced ration. Its high content of calcium carbonate is well known to produce a chemically unique intestinal environment which undoubtedly has some effect upon the character of the intestinal flora and the action of their phytases as well.

In view of the observations of Templin and Steenbock [1933, 1] that immature field maize was definitely less rachitogenic than the seed from which it was produced, P relations in immature maize were compared in Series IV. Large-sized ears of Golden Glow, a yellow dent maize, still in the milk stage and carrying a brown silk on well enclosed cobs, were husked in the laboratory and the kernels cut from the cob by hand. The maize was carefully dried and ground, the consistency of the final product approaching that of flour. It had a sweet agreeable odour. Portions of the original fresh immature maize were suspended in water, preserved with chloroform and toluene, and autolysed for 10 days. Comparisons were made with mature maize obtained from the same plot later. The rations were compounded so as to contain inorganic P comparable in amount with that contained in the immature maize ration. Immature autolysed maize was used in one ration, mature maize supplemented with the proper amount of H_3PO_4 in another. In still another ration phytin was substituted for H_3PO_4 on the basis of equivalent amounts of P.

The results of this series (Table IV) confirm the earlier reports from this laboratory that immature maize is definitely less rachitogenic than mature maize.

Table IV. Series IV. *Relation of inorganic P content to the rachitogenic properties of immature and immature autolysed maize.*

No.	Modification	Ration 2965 Maize component				Animals Each figure is average of 6 rats				
		In- organic P %	Total P %	In- organic % of total	Ca/P ratio	Gain in wt. g.	Daily food con- sumed g.	Femora g.	Ash g.	Ash %
20	Immature maize	0.24	0.39	61	3.4/1	49*	8.5	0.134	0.060	43.5
21	Immature autolysed maize	0.24	0.37	65	3.6/1	50.6	8.5	0.137	0.069	45.2
22	Mature maize	0.02	0.33	6	4/1	44†	8.5	0.101	0.031	31.2
23	Mature maize + H ₃ PO ₄	0.24	0.55	44	2.4/1	48	8.5	0.147	0.070	47.4
24	Mature maize + phytin	0.02	0.55	4	2.4/1	44	8.5	0.111	0.039	34.5

* 1 rat died.

† 2 rats died.

They also demonstrate that this difference can be correlated with a simultaneous rise in inorganic P. Immature maize and immature autolysed maize, both containing the same amounts of inorganic P, show comparable antirachitic effectiveness. Determinations of phytin-P on these samples showed that the increase in inorganic P could be almost entirely accounted for by the hydrolysis of phytin.

In an effort to answer the question whether the unavailability of phytin-P wholly explains the anomalous behaviour of cereal grains or whether there might be other anticalcifying factors present as claimed by Mellanby, the experiments of Series V were devised. Phytin was added to maize in the amount in which it naturally occurs, and then, by acid hydrolysis of the maize in one case and of phytin added to maize in another case, rations with the same inorganic P content were produced. If the hydrolysis of phytin with the corresponding liberation of inorganic P were alone responsible for improved calcification, comparable calcification should be obtained in the two rations. If factors other than phytin were involved and if these yielded to acid treatment along the lines suggested by Green and Mellanby [1928], Holst [1927] and others, it was to be expected that there would be a discrepancy in the results.

In preparing this series of rations, allowance was not made for the hydrolysis of other organic P compounds besides phytin. Consequently, the acid treatment of maize gave rise to a greater amount of H₃PO₄ than had been expected. In order to compensate for this, the treated maize was diluted with mature untreated maize to give a corresponding inorganic P content (Ration 28). To preclude any possibility of a deficiency of vitamin B, incurred by the drying of the maize, 4% of yeast was added to each of the rations in this series, at the expense of the wheat gluten component.

Hydrolysis of the phytin was effected by heating the phytin in 0.05% HCl solution in an autoclave at 15 pounds pressure for 6 hours. The solution was then evaporated at room temperature on the yellow maize component of Ration 2965. This treatment was found sufficient to convert all the phosphoric acid of phytin into the inorganic form.

The maize itself was hydrolysed in essentially the same manner. Sufficient 0.05% HCl was added to produce a thick mash at p_H 4.4. This was heated in an autoclave at 15 pounds pressure for 6 hours. Practically quantitative hydrolysis of the organically bound phosphoric acid was effected. Sodium carbonate in amount calculated to neutralise the HCl was then added and the mixture dried at 43°. The amount of NaCl added later in compounding Ration 2965 was reduced to allow for the amount formed by neutralising the acid mixture.

The results of the ash analyses in this series (Table V) unquestionably confirm the importance of the inorganic P content and point to it as the prime factor in determining the antirachitic properties. The striking similarity in the extent of calcification on Rations 27 and 28 and again on Rations 29 and 30 where the

Table V. Series V. *Effect of acid hydrolysis of maize on its rachitogenic properties.*

		Ration 2965		Animals						
		Maize component		Each figure is average of 6 rats						
No.	Modification	In-organic P %	Total P %	In-organic % of total	Ca/P ratio	Gain in wt. g.	Daily food consumed g.	Femora g.	Ash g.	Ash %
25	Mature maize (basal)	0.02	0.33	6	4/1	49	8.8	0.125	0.045	36.3
26	Untreated maize + untreated phytin	0.02	0.46	4	2.9/1	51	8.8	0.129	0.053	41.2
27	Untreated maize + hydrolysed phytin	0.16	0.46	35	2.9/1	46	8.8	0.150	0.072	48.4
28	Hydrolysed maize diluted	0.16	0.33	48	2.7/1	38	8.8	0.143	0.070	49.0
29	Hydrolysed maize + untreated phytin	0.25	0.46	54	2.9/1	49	8.8	0.173	0.095	55.2
30	Untreated maize + untreated phytin + H_3PO_4	0.25	0.76	33	4/1	52	8.8	0.182	0.099	54.5

inorganic P content was kept constant bear out this contention. The origin of the phosphoric acid appeared to be of no importance. No positive evidence suggesting the existence of unidentified factors involved in the anticalcifying effect of cereals was obtained. We desire, however, to call attention to the slight improvement in calcification observed in Ration 26 with the addition of phytin. This result is not in accord with our earlier findings, where it was entirely without effect. Apparently, a partial utilisation of phytin can occur under certain conditions.

In Series VI, mature yellow maize was hydrolysed in one case by heating with 0.05 % HCl for 2 hours in order to effect only a slight increase in inorganic P, and in another case for 8 hours to effect rather complete hydrolysis with the immediate objective of securing possible differential hydrolysis and destruction of the phytin and the hypothetical toxamin of Mellanby.

The "low inorganic P maize" produced by mild hydrolysis was used as the basis in compounding one ration. Assuming all the inorganic P to have arisen from hydrolysis of phytin, a corresponding amount of phytin was restored to the ration. For comparison, a ration using mature yellow maize to which H_3PO_4 had been added in amount exactly equal to that which had arisen from acid hydrolysis was used. Two other rations were prepared at a higher level of inorganic P, one using the maize which had been hydrolysed for 8 hours, diluted with equal parts of untreated maize, and the other using untreated maize and H_3PO_4 sufficient to equalise the inorganic P content. Phytin was restored to the acid-treated ration in an amount equal to that which had been hydrolysed. Two pairs of rations were compounded in this series, each pair containing identical amounts of both total as well as inorganic P. An odd group using yeast as a source of P was inserted to complete the series. It was fed at a level equivalent in P to a control ration to which H_3PO_4 had been added.

Data obtained from this experiment were expected to afford an insight into the possibility of the existence of the afore-mentioned other factors. Assuming

the phytin-P and inorganic P relations to be the complete explanation, comparable degrees of calcification should be obtained on these rations.

The results of this series (Table VI) on the whole reaffirm our previous conclusions. However, the fact that there occurred a consistent though slight

Table VI. Series VI. *Comparison of the rachitogenic properties of acid-hydrolysed maize with control maize containing same amounts of inorganic and total P.*

		Ration 2965 Maize component				Animals Each figure is average of 6 rats				
		In- organic P %	Total P %	In- organic % of total	Ca/P ratio	Gain in wt. g.	Daily food con- sumed g.	Femora g.	Ash g.	Ash %
No.	Modification									
31	Mature maize (basal)	0.02	0.33	6	4/1	54	8.4	0.118	0.038	32.1
32	Hydrolysed maize + phytin	0.09	0.40	22	3.3/1	59	8.4	0.151	0.067	44.5
33	Basal + H ₂ PO ₄	0.09	0.40	22	3.3/1	60	8.4	0.138	0.060	43.0
34	Hydrolysed maize + phytin	0.16	0.47	34	2.8/1	52	8.4	0.155	0.074	47.3
35	Basal + H ₂ PO ₄	0.16	0.47	34	2.8/1	62	8.4	0.158	0.071	44.5
36	Basal + yeast	0.04	0.40	10	3.3/1	65	8.4	0.137	0.052	43.0

superiority in calcification on the rations which contained acid-treated maize suggested that the state of the phosphorus compounds did not provide the complete answer. Whether the improvement in calcification was due to the destruction of Mellanby's hypothetical toxamin, the hydrolysis of fibre or other changes which can affect calcification, our data do not reveal.

The observed unavailability of phytin P to the rat suggested a study of the action of various enzymes on phytin. Plimmer [1913] found no evidence for the absorption of phytic acid, or for its hydrolysis by enzymes of the intestinal mucosa from a number of animals. Phytic acid was attacked readily by only one enzyme, phytase, which is found principally in bran and the castor bean. Hart *et al.* [1909] concluded that in the pig "when the food supply of P was entirely organic and 80 % of it consisted of phytin, the form of the excreted P was almost wholly inorganic".

We made some observations on the hydrolytic effect of extracts of the intestine from the rat and the chick on phytin as a substrate. No evidence for the enzymic hydrolysis of phytin was obtained even after 9 days' incubation. This suggests that future studies should concern themselves with the phytase activity of the intestinal flora and the influence that dietary changes have thereon.

SUMMARY.

1. Germinated autolysed maize, immature maize, and HCl-treated maize, which had been previously demonstrated to be less rachitogenic than mature maize, were shown to owe this property primarily to an increased content of inorganic P.

2. Treatment of maize with HCl improved its antirachitic properties in proportion to the extent that its phytin was hydrolysed.

3. The inorganic P content of variously treated samples of maize bore a direct relation to the antirachitic effectiveness of the ration and an inverse relation to the phytin content.

4. Phytin proved itself to be a poorly available source of P when fed to the rat in Ration 2965, in contrast with phosphoric acid and sodium glycerophosphate.

5. Acid-treated maize was found to produce slightly better calcification than untreated maize, beyond that which could be accounted for by the increase in inorganic P content. The possible existence of other factors must still be given consideration.

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CLXII. THE DESTRUCTION OF OAK BY THE DEATH-WATCH BEETLE.

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(Received 2 May 1936.)

CERTAIN of the oak timbers of Rothamsted House were recently found to be extensively damaged by wood-boring insects, the damage being ascribed chiefly to the death-watch beetle (*Xestobium rufovillosum*).¹ The timbers are believed to be of great age, and some were probably used in an earlier building. In 1623 the house was extensively altered and the exposed timbering of the original house covered by a brick front, which prevented the damage from being observed before. It is certain, therefore, that the structural timbers are more than 300 years old and probably considerably older. Some of the framing posts supporting the principal rooms were in a serious condition as a result of *Xestobium* attack and had to be replaced. The opportunity was taken of obtaining analyses of this wood and of the borings or frass to which much of the wood had been reduced. In this way some additional information as to the wood constituents utilized by these larvae has been obtained.

EXPERIMENTAL.

Two samples of frass were obtained by shaking two heavily damaged timbers. The material consisted of very fine dust and tiny pellets, together with larger wood fragments which were sieved off separately. The frass was easily reduced to a uniform condition for analysis. The two samples, F 1 and F 2, differed considerably in colour. For comparison a portion of sound wood was also analysed (W 1). Later, owing to differences in composition between F 1 and F 2, a further small piece of damaged timber was selected, the frass shaken out (F 3) and some of the most seriously damaged wood, which was heartwood, cut away with a chisel, separated from frass and ground up (DW 1). The centre portion of the same piece of timber was undamaged and radial shavings were taken and ground up for analysis (W 2). In this way, analyses of frass, damaged wood and sound wood from the same timber have been obtained. The analytical figures are not directly comparable since the loss in weight as a result of attack is unknown, but the differences are of such a nature as to leave no doubt of the changes due to the activities of these larvae (Table I). In view of the importance of the lignin determination on this material and the lack of agreement as to the most suitable method, the results obtained by four procedures are given. Good agreement was obtained, as usual, between the determinations carried out after an acid pre-treatment and by the method of Ritter *et al.* [1932]. In the other procedures, the result is affected by the presence of pentose groupings.

¹ A thorough examination was made by Dr F. R. Cann of the Forest Products Research Laboratory, Princes Risborough.

Table I. *Analysis of Xestobium frass and oak timbers.*

All results expressed on oven-dry material, extracted with alcohol-benzene before analysis.

Determination	From same timber					
	Frass F 1	Frass F 2	Wood W 1	Frass F 3	Damaged wood DW 1	Wood W 2
Cellulose	27.5	31.1	52.6	30.2	32.1	47.2
Total furfuraldehyde	9.8	11.6	14.4	9.2	8.5	13.8
Furfuraldehyde from cellulose	3.5	5.1	9.0	3.2	3.8	7.8
Xylan in cellulose	5.4	7.9	13.9	5.0	5.8	12.1
Furfuraldehyde from hemicelluloses	6.3	6.5	5.4	6.0	4.7	6.0
Lignin ¹ (after hydrolysis)	31.9	24.9	18.7	30.3	28.3	19.8
Lignin ² (direct)	38.9	34.3	24.8	40.3	36.8	27.8
Lignin ³ (direct)	35.3	31.6	22.6	37.9	35.1	25.2
Lignin ⁴ (after extraction)	31.2	25.9	18.2	30.7	27.3	19.5
Hot water-soluble ⁵	19.3	24.1	17.6	19.0	24.3	18.2
Alkali-soluble ⁶	66.9	54.5	31.7	63.7	59.8	42.3
Acid-soluble ⁷	30.0	39.0	33.7	29.6	31.7	32.8

¹ Pre-hydrolysis with 5% H₂SO₄, 1 hour; then 72% H₂SO₄, 16 hours, temp. <20°, diluted to 3% and boiled 2 hours.

² 72% H₂SO₄, 16 hours, temp. <20°, diluted to 3% and boiled 2 hours.

³ 72% H₂SO₄, 2 hours, temp. <20°, diluted to 3% and boiled 2 hours.

⁴ Pre-extraction with hot water, 3 hours; then 72% H₂SO₄, 2 hours, temp. <20°, diluted to 3% and boiled 2 hours [Ritter *et al.*, 1932].

⁵ Extracted in boiling water-bath, 3 hours.

⁶ Extracted with 1% NaOH, in boiling water-bath, 1 hour.

⁷ Extracted with 5% H₂SO₄, at the boil, 1 hour.

DISCUSSION.

The analyses of the frass are so widely different from those of the sound wood as to leave no doubt that there has been an extensive removal of carbohydrate material and notably of the cellulose. Lignin on the other hand is apparently relatively resistant and has consequently accumulated. The cellulose/lignin ratio is perhaps a good indication of the differences in composition (Table II), the lignin figures obtained after pre-hydrolysis being used for this purpose.

Table II. *Cellulose/lignin ratio on sound oak and Xestobium frass.*

	Cellulose/lignin
Frass, F 1	0.86
Frass, F 2	1.24
Sound wood, W 1	2.81
Frass, F 3	0.99
Damaged wood, DW 1	1.13
Sound wood, W 2	2.38

It is curious to note that the xylan in the cellulose was removed to a greater extent than the cellulose taken as a whole. The xylan as % of the cellulose in the sound wood is 25.7 and in the frass from it 16.6. If the assumption be made that the lignin has been entirely unattacked, some indication of the loss of weight undergone by the wood in conversion into frass may be obtained. The loss appears to be about one-third, that is to say 100 g. of oak have lost 34.5 g., of which 27.4 g. were cellulose. The remainder is easily accounted for in the removal of some of the hemicelluloses and of cell contents. The validity of this assumption as to the unavailability of lignin is somewhat questionable. The expedient of comparing the analyses of frass and sound wood by adjustment to

an equal lignin content has, however, previously been used in studies on wood-boring larvae by Ripper [1930], and on *Teredo* by Dore & Miller [1923], and provides a minimum estimate of total loss and cellulose digestion, for if there had been, in fact, some removal of lignin, these figures are an understatement.

The frass is produced by the boring activities of the larvae, the wood being passed through the alimentary system and undergoing the changes noted above as a result of the action of intestinal enzymes and symbiotic bacteria or protozoa. The analyses of the damaged wood (DW I in Table I) are difficult to understand since the changes seem to be extensive and the composition approaching that of the frass. In the sample analysed, the wood was reduced to a mere frail honey-comb structure, the boring having obviously taken place between the rays and through the softer spring wood of the annual rays. The profound nature of the changes in the residual wood suggests that possibly the infected frass as it leaves the larvae might have some local effect on the adjacent wood, continuing as long as moisture relationships permit. Applying the same adjustment to an equal lignin content, the total loss is in the region of 30 %, of which 25 % may be accounted for by the removal of cellulose. These losses are nearly as great as those sustained by the frass.

CONCLUSION.

These results are supplementary to and a confirmation of the observations made by Campbell [1929] and Ripper [1930] on the activities of the larvae of the death-watch beetle, and leave no doubt that the carbohydrates of the cell-wall are extensively utilized, either directly or more probably by the agency of an intestinal micro-flora. Other wood-boring larvae, such as *Lyctus* and *Cossus*, apparently make no use of the skeletal material of the wood, since the frass obtained is almost identical in composition with the sound wood. In so far as it is possible to form an estimate of the material removed by *Xestobium*, the cellulose accounts for 80 % of the total loss, which in these timbers analysed was in the region of one-third of the weight. In most microbiological fermentations with bacteria or fungi, it is unusual to find such a high proportion of the total loss accounted for by the cellulose, and this is an indication of the exceptional activity of the digestive system of these larvae.

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CLXIII. A FILTER OF PYREX GLASS AND STAINLESS STEEL FOR USE WITH SEITZ FILTER-PADS.

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(Received 8 May 1936.)

WE have designed a holder for use with Seitz EK bacterial filter-pads (diameter 3 cm.) in which the filtering liquids come in contact only with pyrex glass and a disc of stainless steel. This apparatus has the advantage that toxic metals (e.g. copper) cannot be dissolved by the filtering liquids as from the ordinary metal holder from which the plating (e.g. silver or rhodium) has been worn. Also the glass filter-tubes can be cleaned with chromic-sulphuric acid mixture; this is essential in much work in bacterial nutrition where traces of active material can produce growth.

The design is shown in Fig. 1, drawn to scale. The pyrex glass filter-tubes A_1 and A_2 (made to our design by Messrs James A. Jobling, Wear Glass Works, Sunderland) are supplied with their flanges ground flat. A shallow recess is

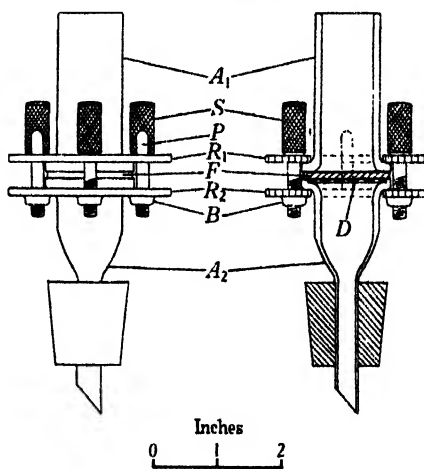


Fig. 1.

ground out of the lower piece, of appropriate diameter and depth to allow a stainless steel disc (D), for supporting the filter-pad (F), to lie in it flush with the flat surface of the flange of the lower glass piece. The disc for supporting the filter-pad is cut from high grade stainless steel and drilled with holes (approximate diameter not greater than $\frac{1}{10}$ in.) over the central portion. The whole disc is burnished, including the filtering holes, to eliminate the risk of corrosion. The

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flat metal clamping rings (R_1 , R_2 ,) are cut from sheet brass (approximately $\frac{3}{8}$ in. thick), the internal diameter being of a size to clear the glass barrel of the filter tubes, but to clasp the glass flanges. The lower clamping ring is supplied with 3 tapped bushes (B) and 3 dowel pins (P), the upper clamping ring having clearance holes to correspond with the tapped holes and pins. The two rings are drawn together by the 3 screws, using finger-tightening by the milled heads (S).

To assemble the apparatus, the filter-pad is placed on the supporting stainless steel disc, the pad itself acting as a gasket between the two glass parts. With the fingers the screws are tightened just enough to hold the filter-pad in place, and the whole apparatus is wrapped and autoclaved for sterilization. For use the filter is fitted aseptically in the usual way to its appropriate sterile filter-flask, the screws are screwed to finger-tightness and the apparatus is then ready.

We wish to thank Dr C. G. L. Wolf for simplifying our original design of the clamping part.

The whole apparatus or parts of it can be supplied by Messrs A. Gallenkamp & Co. Ltd., Sun Street, Finsbury Square, London, E.C. 2.

CLXIV. THE DETERMINATION OF CHLORIDE IN BODY FLUIDS BY DIRECT TITRATION.

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NUMEROUS methods for the estimation of chlorides in body fluids have been published since Mohr [1856] introduced the titration of chloride with silver nitrate using potassium chromate as the indicator, but it was not until comparatively recent years, with Fajans & Hassel's [1923] introduction of adsorption indicators, that any real advance was made in the direct titration of chloride.

The superiority of adsorption indicators over chromate soon established them in general analysis but it was only to a very slight degree that they were used in the estimation of chlorides in body fluids, probably because they have little if any advantage over chromate in the titration of body fluids containing protein.

Saifer & Kornblum [1935] have shown that by using suitable concentrations of organic solvents, small amounts of blood or cerebrospinal fluid and a suitable adsorption indicator the chloride can be titrated directly without removing the precipitated protein.

In the present instance considerable work has been done in endeavouring to find if it would be possible to titrate the chloride directly, without removing or precipitating the protein normally present in body fluids.

In the laboratory of Dr J. Patterson¹ at Charing Cross Hospital a method for the estimation of chloride in cerebrospinal fluid has been in use for some years. Use is made in this method of the fact that if the chloride is titrated at the iso-electric point of the protein the minimum uptake of silver takes place. The chief drawback of the application of this method to biological fluids in general, using chromate as the indicator, is that it is vitiated by protein contents exceeding about 0.6% when 1 ml. of fluid is used. Another drawback is that if the reaction is more acid than pH 5 the silver chromate precipitate does not form to indicate the end-point.

To ascertain whether it would be possible to apply this method in general, using adsorption indicators, a complete study was made of the figures obtained when body fluids were titrated at a definite pH. It was thought that it might be possible to arrive at a point where these fluids could be titrated in an aqueous medium without precipitating or removing the protein or using organic solvents, and consequently larger quantities of fluid could be used if so desired to make the titration less liable to experimental error.

It was subsequently found that this was possible in all body fluids which were tried with the exception of blood, where the protein content is so high as to make the end-point of a direct titration very indefinite.

For the purposes of investigation many adsorption indicators were available but most workers are agreed that fluorescein is the most suitable for the titration of chloride, providing that the concentration of chloride is not below $N/100$ and that the solution is neutral or at the most very feebly acid with acetic acid.

¹ To whom the author wishes to express his thanks for permission to make use of unpublished data.

It has been shown, however, by Kolthoff *et al.* [1929] that dichlorofluorescein, which gives the same colour changes as fluorescein, is not subject even to these limitations since it can be used in very dilute solutions of chloride and is not affected by appreciable concentrations of acetic acid. Kolthoff [1927] and Pieters [1929] have also shown that both bromophenol blue and bromocresol purple among many other colouring matters could be used for this titration under similar conditions.

To show the errors due to the presence of protein when titrating in an aqueous medium using these indicators a whole series of estimations was carried out (Table I). It will be seen that the presence of protein in any appreciable quantity rendered the result higher than was actually true, although the figures obtained were more nearly correct than with chromate as the indicator.

For the purpose of comparison, the chloride was estimated by a back-titration method using the following technique: 1 ml. of fluid was mixed with 5 ml. of concentrated nitric acid in a 6 × 1 in. test-tube and an excess of silver nitrate added. This mixture was then evaporated down to a small bulk over a free flame, cooled and made up to the original volume with distilled water. 6 ml. of acetone were added and a standard amount of iron alum and the mixture was then titrated with alcoholic thiocyanate. A blank determination was carried out under identical conditions and the chloride calculated from the difference.

Table I.

Material	Back-titration figure	Indicator used			Protein %
		Potassium chromate	Fluorescein	Dichloro-fluorescein	
Urine	425	428	Not definite	Not definite	Nil
"	208	216	"	"	"
"	760	765	"	"	"
"	704	780	"	"	0.15
"	436	494	"	"	2.02
Cerebrospinal fluid	706	710	708	705	0.025
"	632	660	659	656	0.320
Milk (human)	104	136	132	124	1.03
Milk (cow's)	183	244	242	236	2.82
Gastric juice	563	622	620	608	0.13
"	380	412	400	396	0.08
"	660	692	690	686	0.24
Blood serum	594	704	692	642	6.72
"	644	700	690	666	5.89
Blood plasma	652	696	682	670	5.76

Urine. From Table I it will be observed that the indicators already mentioned do not give a satisfactory end-point, as there appears to be in urine a substance or substances having greater affinity for adsorption into the precipitated chloride than the fluorescein. The coloured complex which usually forms on the surface of the silver compound with the dye does not occur and as a result there is no visible end-point. Subsequent investigation showed that the actual substance responsible for the lack of adsorption was urea, as the same difficulty was experienced in the analysis of a blood specimen containing 0.7 % of urea. Eosin, a dyestuff often used for the silver titration of bromine, is of no use in an aqueous medium for the estimation of the chloride because it is too strongly adsorbed and competes too successfully against the comparatively feebly adsorbed chloride ion. Owing to this fact it was thought that this dye might be used with urine to give the desired result.

Experimentally, it was found, however, that this reasoning was incorrect, but that a mixture of the two dyes, eosin and dichlorofluorescein, could be used, giving a very definite and sharp end-point.

The technique used for the estimation of chloride in urine is as follows.

Take 5 ml. of urine and shake with 0.1 g. of decolorizing charcoal and filter. As a general rule take 1 ml. of the filtrate, but in cases where the chlorides are likely to be low a larger quantity is desirable. Add this to 10–15 ml. of distilled water and then add 1 ml. of eosin and 2 ml. of dichlorofluorescein. Titrate with silver nitrate until the precipitate takes on a bright magenta hue. Calculation is made on the basis that 1 ml. of silver is equivalent to 2 mg. of sodium chloride. The strengths of the reagents used are tabulated at the end of the paper.

From Table II it will be seen that this technique although suitable for a straightforward titration of chloride did not help in regard to obtaining an accurate estimation in the presence of protein.

Table II.

Material	Back-titration figure	Indicator used		Protein present %
		Potassium chromate	Mixed eosin and dichlorofluorescein	
Urine	425	428	425	Nil
"	208	216	206	Nil
"	760	765	760	Nil
"	704	780	760	0.15
"	436	494	476	2.02

It was next decided to try whether accurate results could be obtained by titrating at the isoelectric point of the protein. Furthermore, to ascertain whether dichlorofluorescein could be used at various pH values and so take the place of chromate in the titration, a series of buffer solutions as recommended by Cole [1926] was used as the medium in which a definite amount of chloride was titrated.

Throughout the whole of the range, pH 3.8–5.6, the end-point was constant and definite using the dye as the indicator but with chromate it was soon apparent that the end-point became more indistinct as the concentration of acid became higher.

Dichlorofluorescein not being affected by the variation in pH between the figures mentioned, it was experimentally possible to determine whether by using the mixed indicator a point could be attained at which the uptake of silver would be negligible and an accurate determination could be obtained.

The same technique was used as already described, but 5 ml. of the buffer solution were added to each of the titration solutions.

As the following tables show the minimum uptake of silver takes place when the buffer used is at pH 5, although this does not necessarily mean that the solution

Table III.

Material	Back-titration figure	Potas-sium chromate	Indicator used					Protein present %
			Mixed eosin and dichlorofluorescein					
			Aqueous medium	pH 4.6	pH 4.8	pH 5.0	pH 5.25	
Urine	425	428	425	425	426	424	424	Nil
"	208	216	206	208	206	207	208	Nil
"	760	765	760	758	758	760	760	Nil
"	704	780	760	700	706	704	722	0.15
"	436	494	476	432	440	436	448	2.02

is itself at pH 5 for the addition of certain body fluids would make a considerable difference in the pH.

Cerebrospinal fluid, gastric juice and milk. Using the same technique of titrating at a definite pH, Table IV shows that very little variation exists between these fluids in regard to the best point of titration, milk giving more accurate results if titrated at a slightly lower pH, although for practical purposes this can be ignored.

The most satisfactory technique to use for the analysis of these fluids is as follows. In a 6 x 1 in. test-tube place 15 ml. of distilled water. Add 5 ml. of buffer solution to bring to the required pH. Then add 1 ml. of milk, cerebrospinal fluid, or filtered gastric juice and 2 ml. of dichlorofluorescein and titrate with silver nitrate until the precipitate formed is coloured a definite pink.

Calculation: ml. of silver nitrate used multiplied by 200 = mg. NaCl/100 ml.

Table IV.

Material	Back-titration figure	Potassium chromate	Indicator used					Protein present %
			Aqueous medium	Dichlorofluorescein				
				pH 4.6	pH 4.8	pH 5.0	pH 5.25	
Cerebrospinal fluid	706	710	705	706	706	706	706	0.025
"	632	660	656	634	634	632	636	0.320
Milk (human)	104	136	124	108	104	106	118	1.03
Milk (cow's)	183	244	236	180	183	184	212	2.82
Gastric juice	563	622	608	560	564	562	564	0.13
"	380	412	396	380	384	380	384	0.08
"	660	692	686	662	662	660	664	0.24

Blood (serum or plasma). Since blood is very rich in protein it was expected that more difficulty would be experienced in evolving a method which would give comparable results.

Dichlorofluorescein was unsatisfactory for use with blood as at a pH between 4.6 and 5.25 and in the presence of a comparatively large amount of protein the characteristic fluorescence was absent and the end-point was very difficult to determine.

Eosin could not be used because it was too strongly adsorbed and made the end-point even more difficult to determine.

These facts led to the suggestion that if another dye could be used, say a blue dye, which would not play a part in the reaction, but merely make a background for the pink colour of the fluorescein on the silver chloride, the end-point might be more readily detectable.

Numerous dyes were tried and the most satisfactory in use proved to be bromocresol green. The use of this dye, however, did not completely solve the problem as the recognition of the actual end-point required a fair amount of experience on the part of the operator.

Smirk [1927] made use of acetone to stabilize the end-point of the back-titration of chloride with thiocyanate.

It was found that with a little experience a definite end-point could be obtained using serum or plasma in presence of acetone and an absorption indicator, but the figures obtained were about 5-10% on the high side. The use of bromocresol green was unnecessary unless bile was present in the fluid concerned, when the addition of 0.15 ml. of the dye made the end-point more easily detectable.

The technique that was employed is given here, because it was found that by using it, the error introduced in the estimation of milk chlorides with buffer of pH 5.0 could be eliminated.

In a 6 × 1 in. test-tube place 15 ml. of distilled water, 5 ml. of buffer solution, 6 ml. of acetone. Then add 1 ml. of fluid and 2 ml. of dichlorofluorescein. Titrate with silver nitrate until the precipitate formed is coloured a faint pink.

Calculation: ml. of silver used × 200 = mg. NaCl/100 ml.

When the protein was removed by tungstic acid or zinc hydroxide and the filtrate used for the estimation, the same difficulty was apparent as in the case of urine, no visible end-point being obtained. Using alcohol or acetone the end-point was very satisfactory but the result was again high probably owing to evaporation of the fluid.

Since the presence of acetic acid did not appear to have any effect on the analytical result, it was decided to try whether this substance alone could be used for the precipitation of the protein.

Several quantities were tried but difficulty was experienced in ranging the amount to give constant results; when the solution was buffered between pH 4.6 and 4.7 much more satisfactory results could be obtained.

When the usual quantity of fluid, namely 1 ml., was taken and a portion of the filtrate used, in most cases the analysis was very satisfactory. If, however, the chloride content was small and the amount of urea large the results obtained were very erratic.

It will be readily observed that if the amount of filtrate taken is small (actually it is possible to arrange the conditions so as to obtain the equivalent of 0.75 ml. of blood) and the amount of chloride is small, the silver chloride formed during the titration is correspondingly meagre. Consequently, there is not a great deal of precipitate available for the formation of the pink complex and since urea tends to prevent the adsorption of the dye, the combination of these disadvantages obscures the end-point.

Attempts to solve this problem by means of different indicators were not very successful but it was thought that if a standard quantity of chloride were added and the mixture diluted and titrated, the existing conditions would then be approximately normal in the relationship between the chloride and urea.

In practice this arrangement was found to be very satisfactory, and as Table V shows, even when the conditions were such that the urea was present in the maximum amount likely to be encountered and the chloride very low, the agreement between the method described and the standard method was very good.

The technique used is as follows.

In a test-tube place 3 ml. of distilled water, 3 ml. of 0.2 *N* sodium acetate, 3 ml. of 0.2 *N* acetic acid and 1 ml. of serum or plasma. Mix and place in a boiling water-bath for 2 min. Cool and filter through a 7 cm. chloride-free filter-paper. In a 6 × 1 in. test-tube place 7.5 ml. of the filtrate, and add for each 2.5 ml. of the filtrate used 1 ml. of 0.2 *N* sodium acetate. Then add 10 ml. of distilled water and 2 ml. of dichlorofluorescein and titrate in the usual way.

Calculation:

$$\frac{\text{ml. of silver nitrate used} \times 200 \times 4}{3} = \text{mg. NaCl/100 ml.}$$

If, however, the blood is suspected of having a high urea content, or if less than 7.5 ml. of filtrate can be obtained, it is advisable to add before titration 1 ml. of 1% sodium chloride. A blank titration can then be carried out using 10 ml. of buffer pH 5.0, 10 ml. of distilled water, 1 ml. of 1% sodium chloride and 2 ml. of indicator. The blank figure is then subtracted from the unknown figure and calculation is made as previously described.

Table V.

Specimen	Back-titration figure	Direct titration using 7.5 ml. of filtrate	Titration using blank method	Urea content mg./100 ml.
I	612	613	612	35
II	596	598	598	54
III	496	502	498	182
IV	510	522	512	496
V	420	End-point poor 436	416	725

Whole blood. When the analysis of whole blood was attempted using the same technique as for serum or plasma difficulty was experienced on account of the colour. The filtrate obtained was a deep brown, probably owing to the fact that, the precipitation medium being definitely acid, it carried through some of the hæmatin in a soluble form. This led to the suggestion that it might be possible to precipitate the proteins at a higher pH, and so avoid the concentration of acid being strong enough to dissolve the hæmatin.

In practice it was found that, if the precipitation were accomplished between pH 5.25 and 5.5, the filtrate was in the majority of cases quite clear and in the other cases very faintly brown. By using the filtrate obtained, adjusting back to pH 5.0 and estimating the chloride by the blank method described for serum, very accurate results could be obtained.

To obtain figures for comparison, it was necessary to precipitate the proteins before attempting to analyse the chloride content by the back-titration method already described.

The actual technique used for the direct titration was as follows.

Introduce into a test-tube 9 ml. of buffer solution, pH 5.3, add 1 ml. of blood, mix and heat in a boiling water-bath for 2 min. Cool and filter through a 7 cm. chloride-free filter-paper. Take as much of the filtrate as is available and for each 2.5 ml. add 0.3 ml. of 0.2 N acetic acid. Add 1 ml. of 1% sodium chloride, 10 ml. of distilled water and titrate in the usual fashion.

Calculate:

$$\frac{(\text{ml. of AgNO}_3 \text{ used in titration} - \text{ml. used in blank}) \times 2000}{\text{No. of ml. of filtrate used}} = \text{mg. NaCl/100 ml.}$$

Table VI.

Specimen	Back-titration figure	Titration using blank method	Urea mg./100 ml.
I	520	522	32
II	512	513	76
III	402	402	142
IV	310	308	725

Reagents.

Acetone A.R. The acetone used should be first tested to ensure that no precipitate is given with silver nitrate.

Bromocresol green. 0.1 g. is dissolved in 14.3 ml. of 0.01 N NaOH, and diluted to 100 ml. with distilled water.

Dichlorofluorescein. 5 mg. are dissolved in 100 ml. of distilled water containing 2.5 ml. of 0.01 N NaOH.

Eosin. 10 mg. are dissolved in 100 ml. of distilled water.

Sodium acetate. Acetic acid solutions prepared according to Cole [1926] and mixed in varying amounts to give the respective buffer solutions.

Standard silver nitrate. Dissolve 5.812 g. of pure A.R. silver nitrate in distilled water and make up to 1 litre.

SUMMARY.

1. A quantitative method is described for the accurate estimation of chloride by direct titration, using adsorption indicators, in all body fluids not containing protein in excess of 3 %.

2. After removing the protein by suitable means the same method has been adapted to give accurate results in all body fluids where the protein concentration exceeds 3 %.

I wish to express my thanks to Dr E. N. Allott for his criticisms and advice during the course of this work.

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CLXV. REDUCTION OF BILIVERDIN TO BILIRUBIN IN TISSUES.

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THE object of this paper is to investigate whether or not it is possible to regard biliverdin as the primary product of blood pigment breakdown in the body, and bilirubin as arising by reduction of biliverdin. By the combined action of molecular oxygen and of reducing substances like hydrazine hydrate [Warburg & Negelein, 1930] or ascorbic acid [Karrer *et al.* 1933] haemin is transformed into "green haemin". This substance has been proved by Lemberg [1935] to be a bile pigment haemochromogen, and it has been shown that it is easily convertible into biliverdin. It has been suggested that a similar mechanism is at work in the transformation of blood pigment into bile pigment in the body, leading to biliverdin. Since, however, bilirubin is the only, or the predominant, pigment in the bile of many animal species this hypothesis can be correct only if it can be shown that biliverdin is reduced to bilirubin in the body at a rate higher than the rate of blood pigment decomposition.

Little is known about reduction and oxidation of bile pigments in the tissues. When biliverdin is found in bile, its presence is sometimes due to a post-mortem oxidation of bilirubin in the bile exposed to atmospheric oxygen. Dastre & Floresco [1897] have claimed that oxidases play a role in this process. On the other hand, Haycroft & Scofield [1890] observed that the green bile pigment of ox bile became reduced to bilirubin when the bile was kept in tall cylinders. This reduction occurred in the absence of microorganisms, although it was accelerated by putrefactive bacteria. Reduction of biliverdin by liver tissue has been studied by Barry & Levine [1924] who report that at pH 7.4-7.6 biliverdin is reduced by the liver, whereas oxidation of bilirubin is favoured by pH 6.5. Glucose was found to accelerate the reduction. Although biliverdin can doubtless be formed in bile by post-mortem oxidation of bilirubin, this does not explain all findings of biliverdin in bile. It is also present in some cases as the primary bile pigment. Fresh frog's bile often contains green bile pigment and Nisimaru [1931] found that bile containing biliverdin is excreted by the liver of the bull frog when the liver is transfused with blood. Stern [1936] has obtained biliverdin as a by-product in the preparation of catalase from horse liver. The bile of the starving dog is green [Hoppe-Seyler & Thierfelder, 1924]. So is occasionally the bile of *Echidna*² and of the fowl. We have isolated small amounts of biliverdin from fresh rabbit's bile in which bilirubin predominates. In the bile of the guinea-pig we have never observed biliverdin, and for this reason we have used this animal preferably for our experiments.

Pure crystalline biliverdin has not previously been reduced to bilirubin either *in vitro* or by action of tissues. Barry & Levine used the crude mixture of oxidation products obtained by autoxidation of bilirubin in alkaline solution. Fischer *et al.* [1932] subjected mesobiliverdin ("glauco bilin") to reduction with

¹ Working under the J. E. Rofe bequest.

² We owe thanks to Dr Abbie of Sydney University, for giving us three fresh specimens of *Echidna* gall bladders, two of which contained biliverdin.

zinc dust but were unable to identify the product of reduction with meso-bilirubin. For this reason we have also included a study of the reduction of biliverdin and of mesobiliverdin by chemical means.

Since bile pigment is formed to a considerable extent in the liver and is also excreted by this organ, we have particularly studied the reduction of biliverdin in guinea-pig liver, but we have also made a few experiments with other organs of the guinea-pig and with the livers of other animals.

EXPERIMENTAL.

Reduction of biliverdin and of mesobiliverdin by zinc dust.

Fischer *et al.* [1932] transformed glaucobilin (mesobiliverdin) into meso-bilirubin by heating it above the melting-point, but they were unable to obtain mesobilirubin from it by reduction with zinc dust in hot methyl alcohol. This reaction gave a different compound. We have carried out the reduction at room temperature in ammoniacal solution, excluding atmospheric oxygen, since the zinc salt of mesobilirubin is readily oxidized by molecular oxygen. The reduction proceeds in a few minutes under these conditions, whereas Fischer boiled in methyl alcohol for 4 hours.

The reaction was carried out in the apparatus described by Lemberg [1935, p. 1331]. The small dropping funnel contained a solution of 20 mg. mesobiliverdin in 5 ml. 2% ammonia, the tube with the porous filter 1 g. of zinc dust and the small receiver 1 ml. of 20% HCl. During the reduction in the tube with the porous glass plate nitrogen was passing in from below the porous plate agitating the zinc dust. The end of the reduction, indicated by the change of the colour to a clear orange-yellow, was reached in 5 min. The solution was now sucked off from the zinc dust into the HCl which precipitated the pigment. By washing with water, hot glacial acetic acid, then again with water and methyl alcohol and by recrystallization from chloroform mesobilirubin was obtained in both needles and rhombic plates.

Mr Mellor has kindly compared these crystals with those of mesobilirubin prepared by catalytic hydrogenation of bilirubin, and has found them identical in shape, angles, angles of extinction and pleochroism.

Biliverdin was prepared as described by Lemberg [1932; 1934, 1]. It was reduced in the same way to bilirubin. Orange rhombic plates of the characteristic form were produced.

Reduction of biliverdin in animal tissues.

Technique. The animal was killed and bled, and the organs were removed under aseptic conditions, immediately after the death of the animal, and put into sterile Ringer solution. Slices of liver and kidney were prepared according to Warburg's method, the other tissues being finely chopped. 0.3–0.5 g. of the tissues was used for each experiment.

"Liver brei" was obtained by grinding chopped liver in a mortar with the minimum of HCl-extracted sea sand.

"Washed brei" was obtained in the following way.

The liver brei was thoroughly ground with 5 vol. of distilled water in a mortar, and the suspension was kept in the ice-chest for 1 hour. After centrifuging, the tissue was washed 5 times on the centrifuge, each time with about 5 vol. of distilled water.

The first two centrifugates, consisting of a red-brown cloudy solution, were kept as "washings".

The "liver enzymes" preparation mentioned below was obtained from the washings by precipitating the enzymes by half-saturation with ammonium sulphate, filtering the precipitate and dissolving it in 0.2 *M* phosphate buffer pH 7.6. It was then dialysed for 24 hours against 0.1 *M* phosphate buffer of the same pH in the ice-chest. In a few experiments separately mentioned, phosphate buffers were used instead of water for the extraction of the enzymes from the brei. The amount of "washed brei", "washings" and "enzyme preparation" used, always corresponded to about 0.3–0.5 g. liver.

Bacterial infection was avoided by sterilizing all vessels and solutions. Toluene was added regularly, after the first experiments had shown that it had no effect on the reduction. Very few experiments had to be discarded because of bacterial infection.

For each experiment, unless otherwise mentioned, 3 mg. of pure crystalline biliverdin dissolved in 0.1 ml. 0.2 *N* NaOH were used and the total volume of the mixture was 7 ml.

The following buffer media were used:

Ringer solution, mammalian Ringer-bicarbonate (according to Warburg [1930, p. 122]), 0.2 *M* secondary phosphate, 0.2 *M* phosphate buffer pH 7.6. In the experiment at pH 6.5 (Exp. 2, Table I) the pH was adjusted by adding 0.2 *M* phosphate buffer pH 6.0 until pH 6.5 had been reached.

Little or no difference was found in using the different media (cf. Table I).

The aerobic experiments were carried out at 37° by shaking the tissues in buffered biliverdin solutions in 50 ml. Erlenmeyer flasks with air. The qualitative anaerobic experiments were not done under strictly anaerobic conditions. The tissues were incubated at 37° with the buffered biliverdin solutions covered with a 2 in. layer of liquid paraffin. In the quantitative anaerobic experiments the reaction was carried out in vacuum tubes at a low pressure of nitrogen freed from oxygen by passage over heated copper.

Quantitative isolation of the bile pigments from the tissues is impossible. A nearly quantitative extraction followed by separation of bilirubin from biliverdin could, however, be accomplished and is described below. It identifies the yellow pigment formed as bilirubin in each case and gives a fairly accurate account of the rate of reduction, but it is rather time-consuming.

A good rough measure of the degree of reduction can be obtained in a very simple way in all experiments in which heat-coagulated proteins are not present by observing the change of colour from the blue-green biliverdin to the orange-yellow bilirubin. In the experiments with the liver enzymes, the degree of colour change of the solution corresponds satisfactorily with the degree of reduction. In experiments with slices and brei, microscopic observation is applied because the reduction is almost completely limited to the tissue. In the tables, "no" signifies blue-green colour and no reduction, ± green colour and weak or doubtful reduction, + signifies a distinct reduction with change of colour from blue-green to yellowish green, ++ a greenish yellow colour corresponding to about 50% reduction or more, and +++ yellow colour and more than two-thirds to complete reduction.

A further indication of the degree of reduction, particularly in the tissues, is given by the crystallization of bilirubin. With a few exceptions bilirubin crystals were seen whenever there was a +++ reduction, in some cases even with a ++ reduction. Typical "haematoidin" crystals of both forms, rhombic plates and fine needles, were observed. In the tissue slices crystals were generally found lying within the liver cells. It was noted that the parenchymal cells of guinea-pig liver did not display a uniform behaviour to the biliverdin solutions. Some

of the cells were found to refrain from taking up bile pigment and to remain colourless, whereas other cells stored bile pigment. There were slight differences in the degree of reduction in different parts of the tissue, but less marked than the differences in bile pigment storage.

Table I. *Aerobic reduction of biliverdin in guinea-pig liver slices.*

0.4 g. liver slices. 3 mg. biliverdin. Total volume: 8 ml.

Shaken at 37° in Erlenmeyer flasks with air.

Exp. No.	Guinea-pig	Medium	Additions	Reduction after				
				15 min.	1 hour	2 hours	4 hours	16 hours
1	21	Ringer-phosphate pH 7.4	—	—	—	+	+++, crystals	+++, crystals
2	21	Ringer-phosphate pH 6.5	—	—	—	+	+++, crystals	+++, crystals
3	21	Ringer-phosphate pH 7.4	0.1 ml. toluene	—	—	+	+++, crystals	+++, crystals
4	21	Ringer-phosphate pH 7.4	10 mg. thymol	—	—	+	++, crystals	++, crystals
5	22	Ringer	0.1 ml. toluene	—	+	—	+++, crystals	+++, crystals
6	22	Ringer-bicarbonate	„ „	—	+	—	+++, crystals	+++, crystals
7	22	Ringer-phosphate pH 7.4	„ „	—	+	—	+++, crystals	+++, crystals
8	30	Ringer-bicarbonate	„ „	—	—	—	++	+++, crystals
9	41	Ringer-bicarbonate	„ „	—	+	+	++	±
10	41	0.2 M secondary phosphate	„ „	—	+	+	++	+
11	38	Ringer-bicarbonate	„ „	+	+++, crystals	—	+++, crystals	+, a few crystals
12	38	Ringer-phosphate	„ „	+	+++, crystals	—	+++, crystals	+++, crystals
13	38	Ringer-bicarbonate	1 ml. 0.02 M KCN	±	+++, crystals	—	+++, crystals	+
14	38	Ringer-bicarbonate	0.7 g. urethane	+++	+++	—	±	No
15	39	Ringer-bicarbonate	Toluene	+	+	—	++, a few crystals	++
16	39	Ringer-bicarbonate	Toluene + 50 mg. glucose	+	++, a few crystals	—	+++, many crystals	+++, many crystals

The experiments in Table I show that there is considerable aerobic reduction of biliverdin in guinea-pig liver slices. In Exps. 1–10 the guinea-pigs were kept on the normal stock diet. Guinea-pig 38 (Exps. 11–14) had been well fed on carrots 2 days before it was killed, guinea-pig 39 (Exps. 15 and 16) had been starved over the same period. The results show a distinct variation in the speed of aerobic reduction in normally fed animals. In general almost complete reduction in the tissue with crystal formation was found after 4 hours' incubation (e.g. in animals 21 and 22). In a few other cases, however, the reduction was slow (Exp. 8) or incomplete (Exps. 9, 10). In the latter case there is even less reduction than in the starved animal liver so that it is doubtful whether the liver

of the animal 41 can be considered as normal; no visible abnormality, however, was noticed.

There is a very marked influence of the feeding of the animals on the rate of reduction as shown by a comparison between Exps. 11 and 15. Whereas the liver slices of the well fed animal reduce almost completely in 1 hour, and those of the normally fed ones in 4 hours, there is in the starved liver a ++ reduction only, with the formation of a few crystals, after 4 hours. Exp. 16 shows that this difference is due to the lack of substrate, probably glycogen, in the starved liver, since addition of glucose renders the starved liver almost as active as the well fed liver. This confirms the observation of Barry & Levine, that glucose increases the reduction of biliverdin.

Reoxidation after 16 hours, sometimes even with disappearance of bilirubin crystals, is observed occasionally (Exps. 9, 10, 11, 13, 15). A green fringe surrounding otherwise yellow tissue was often observed after 16 hours' incubation, even when the tissue had been uniformly yellow after 4 hours. It will be discussed below whether the tissue plays a role in this reoxidation.

A comparison of Exp. 2 with Exp. 1 and of Exps. 10 and 9 shows that the reduction varies little with the pH over the range 6.5-9. The finding of a strong reduction at pH 6.5 is not in accordance with the result of Barry & Levine mentioned above.

There is no appreciable influence of toluene (Exp. 3) or of thymol (Exp. 4), or of the medium; 1/400 *M* KCN does not inhibit. Less clear is the influence of urethane. It has no action of its own on bilirubin, but in presence of liver it produces almost instantaneous reduction. No bilirubin crystals are observed, and the yellow colour soon gives place again to the green colour of biliverdin so that the reduction has almost disappeared after 4 hours. A satisfactory explanation of this observation cannot be given. It might be that urethane acts by way of protein denaturation which produces a similar effect.

In order to determine the velocity of reduction of biliverdin under aerobic conditions 1 g. liver slices of the guinea-pig 22 was incubated for 4 hours at 37° with 10 ml. Ringer-bicarbonate solution containing 5 mg. biliverdin. The slices at the end of that time were completely yellow and displayed many bilirubin crystals, but no trace of green pigment. They were removed from the solution, the latter was centrifuged and its biliverdin content was determined colorimetrically against a freshly prepared biliverdin standard in Ringer-bicarbonate. 2.3 mg. of biliverdin were found in the solution, so that 2.7 mg. had been taken up by 1 g. liver tissue and had been reduced in 4 hours.

This reducing power is certainly sufficient to account for the total output of bilirubin in the guinea-pig. Provided that the activity of human liver tissue is the same the total reduction to bilirubin of the daily amount of biliverdin (about 400 mg.) can be brought about by 25 g. of liver tissue, about 1/60 of the total weight of the human liver. The reducing power of the tissue is probably much greater even, since the limiting factor of bilirubin formation in the above experiment was clearly the uptake of biliverdin by the cells from the solution, and not its reduction.

In human liver the reduction varied with the time interval between death and autopsy, so that the true rate of reduction could be properly determined. A liver excised 6 hours after death gave a strong aerobic reduction (cf. Table II) which, however, was not complete. It is impossible to recover the bile pigments sufficiently quantitatively from liver slices, and for this reason no quantitative determination of the rate of aerobic reduction could be made. In the anaerobic experiment (cf. Table V) the reduction in the tissue was almost complete after

Table II. *Aerobic reduction of biliverdin in liver slices of different animal species.*

0.4 g. liver slices. 3 mg. biliverdin. 9 ml. Ringer-bicarbonate.
Toluene. Shaken at 37° in Erlenmeyer flasks with air.

Exp. No.	Liver of	Reduction after		
		2 hours	4 hours	16 hours
1	Man	++	+	+
2	Horse	+	++	++
3	Cat	+	—	++
4	Rat	+	—	+++ , many crystals
5	Mouse	++	++ , crystals	+
6	<i>Echidna</i>	++	++†	—
7	Fowl	+	++	++
8	Pigeon	++	++	++
9	Frog	±	—	±

Remarks: * ++ with lactate. † ++ crystals with lactate. ‡ +++ crystals with alcohol.

16 hours. With 1 g. of liver slices, 6 mg. of biliverdin had been incubated, of which at least 3 mg., probably 4 mg., had been reduced. This shows that certainly less than 90 g. of human liver tissue are sufficient for the reduction of the daily bile pigment output; for the reason mentioned above the real reducing power is probably greater.

Aerobic reduction of biliverdin was found in the liver slices of all animals examined, but no more than one or two experiments have been carried out with each species. Only in the livers of the mouse and the rat, and in pigeon and *Echidna* livers in presence of substrate was formation of bilirubin crystals observed.

The human liver was obtained 6 hours after death from pneumonia.

Table III. *Aerobic reduction of biliverdin in different tissues of the guinea-pig.*

Guinea-pig 38	Reduction after		
	1 hour	4 hours	20 hours
Liver	+++ , crystals	+++ , crystals	+, a few crystals
Kidney	+	+	++
Brain	+	++	No
Spleen	+	+	++
Muscle	—	—	No
Heart	—	—	No
Skin	—	—	No
Lung	—	—	No

Under aerobic conditions reduction is found in the liver and also, although less, in the kidney, in the spleen and in the grey matter of the brain. In the skin a thin layer under the epidermis showed a slight (+) and the hair sheath a marked (+++) reduction. Biliverdin might be useful for histological investigations.

Oxidation of bilirubin in tissues.

Some experiments recorded in Table I indicate that on prolonged shaking of the tissues with air bilirubin disappears again from liver slices and is reoxidized to biliverdin, the degree of this reoxidation being found to vary considerably. Reoxidation is perhaps due to exhaustion of reducing substrates, since addition of glucose to starved liver slices prevents disappearance of bilirubin (cf. Exp. 16,

Table I) which was, however, found in both well-fed and starved livers. It was therefore investigated whether liver and other tissues can accelerate the oxidation of bilirubin.

Table IV. *Oxidation of bilirubin in presence of different tissues of the guinea-pig.*

3 mg. bilirubin in 9 ml. Ringer-bicarbonato. 0.3 g. tissue slices or finely chopped tissue. Toluene. Shaking for 20 hours at 37° in Erlenmeyer flasks.

		Oxidation after 20 hours
Liver	Tissue yellow, bilirubin crystals, solution greenish yellow	Less than control
Kidney	Tissue slightly greenish, solution olive-brown	= Control
Spleen	Tissue partly green	Slightly more than control
Lung	Tissue slightly greenish	Slightly more than control
Muscle	Fibres greenish, edges of fibres and connective tissue yellow	In the whole = control
Heart	Green on surface of tissue	More than control
Skin	Green spots in tissue, edges brown	= Control
Blood	No biliverdin found	No oxidation
Control	Solution olive-brown	—

Table IV shows that only heart accelerates the oxidation of bilirubin distinctly, and spleen and lung to a minor degree. Kidney, muscle and skin show very slight, if any, acceleration whereas liver tissue retards the oxidation. Even washed liver brei still showed this retardation. If there is any oxidation of bilirubin in the liver, it is very slow. Hence, the biliverdin-bilirubin system cannot play a catalytic role in the respiratory mechanism of the liver cell.

No oxidation of bilirubin was found in sheep's blood. After the erythrocytes had been removed by centrifuging the serum proteins were precipitated with 2 vol. of alcohol. The solution was yellow. The pigments were extracted with ether. 1% HCl failed to remove any green pigment from the yellow ethereal solution, which proves the absence of biliverdin.

Anaerobic experiments with liver slices.

The slowness of reoxidation of bilirubin in the liver of the guinea-pig is also indicated by the rather small difference between the rates of biliverdin reduction under aerobic and anaerobic conditions. This will be seen from a comparison of Exp. 1, Table V with Exp. 9, Table I, or of Exp. 4, Table V with Exp. 1, Table I. The somewhat greater difference between anaerobic and aerobic reductions in the livers of other animal species (cf. Table V with Table II) might either indicate a more rapid reoxidation of bilirubin in these livers or, more probably, inhibition of the reduction of biliverdin by oxygen competing with biliverdin as hydrogen acceptor in those livers which contain a small store of hydrogen donors. Like the aerobic, the anaerobic reduction of biliverdin is not inhibited by cyanide even in high concentration (Exps. 6 and 7), and is accelerated by glucose (Exp. 2).

Table VI shows that almost all tissues of the guinea-pig are capable of reducing biliverdin under anaerobic conditions, skin being an exception. In the skin reduction was observed only in those places mentioned above.

Biliverdin was not reduced by incubation with defibrinated sheep's blood at 37° for 24 hours. The blood was worked up in the following way. Erythrocytes were removed by centrifuging. From the green serum the proteins were precipitated with twice the volume of alcohol, and the centrifugate was distributed

between ether and water in the manner described below. Biliverdin was completely extracted from the ether with HCl.

Table V. *Anaerobic reduction of biliverdin in liver slices.*

3 mg. biliverdin in 9 ml. Ringer-bicarbonate. 0.4 g. tissue. Toluene. Under liquid paraffin.

Exp. No.	Liver of	Medium	Reduction after			
			1 hour	2 hours	4 hours	16 hours
1	Guinea pig 41	—	+	++	++	+++
2	„ 41	0.03 M glucose	+++	+++, a few crystals	—	+++, many crystals
3	„ 78	—	—	+	++, crystals	++, crystals
4	„ 21	—	+	++	+++, crystals	+++, crystals
5	„ 339	—	—	—	—	+++, crystals
6	„ 339	0.01 M KCN	—	—	—	+++, crystals
7	„ 339	0.1 M KCN	—	—	—	+++, crystals
8	Man	—	+	—	—	+++, crystals
9	Horse	—	—	++	+++	+++
10	Cat	—	—	++	++	++
11	Rabbit	—	No	—	—	+++, crystals
12	Rat	—	—	++	—	+++, crystals
13	Mouse	—	++	+++	+++, crystals	+++, crystals
14	<i>Echidna</i>	—	—	+++	—	+++, many crystals
15	Fowl	—	—	++	+++	+++, crystals
16	Pigeon	—	—	+	++	+++, many crystals
17	Frog	—	+	—	—	++, a few crystals

Table VI. *Anaerobic reduction of biliverdin in different tissues of the guinea-pig.*

3 mg. biliverdin in 9 ml. Ringer-bicarbonate. 0.4 g. tissue. Toluene. Under nitrogen at 37°.

	Reduction after	
	2 hours	20 hours
Liver	++	+++ , crystals
Kidney	+++ , a few crystals	+++ , crystals
Brain (grey matter)	+++ , a few crystals	+++ , crystals
Spleen	No	+++ , crystals
Muscle	No	+++ , crystals
Heart	No	+++ , crystals
Lung*	No	+++ , crystals
Skin	No	No

* Lung gives a reduction only if air is carefully removed from the tissue by repeated evacuation and filling with nitrogen.

Experiments with liver brei.

Liver brei reduces biliverdin as readily as liver slices (Exp. 2, Table VII); this reduction differs from that observed with slices in being extended to the solution. Cyanide (1/400 *M*) does not inhibit the reduction (Exp. 3). Careful washing of the brei with water renders it almost inactive (Exp. 4), and addition of the washings restores its activity (Exp. 5). This restored system now causes a stronger reduction in the solution than in the tissue. Exps. 6 and 7 show that washing with distilled water removes not only substrates but to a large extent also the enzymes responsible for the dehydrogenation of biliverdin. That the reduction in Exp. 6 is not due to heat-stable reducers in the washings, at least to any great extent, is proved by Exp. 8 with the washings of heated brei. The precipitate obtained by half-saturation of the washings with ammonium sulphate contains the enzymes; it is spoken of subsequently as "liver enzymes". It is almost without reducing power either alone (Exp. 9) or together with the washed liver brei (Exp. 10). This is due to the lack of substrates, which remain in the filtrate from the ammonium sulphate precipitate. The activity is restored by the addition of heated, filtered washings (Exp. 11) or by the addition of suitable substrates as will be seen below.

Table VII. *Anaerobic reduction of biliverdin in liver brei and influence of washing and heating.*

3 mg. biliverdin in 9 ml. Ringer-bicarbonate. In test-tubes under liquid paraffin at 37°. Toluene. Sol.=reduction in solution.

Exp. No.	Guinea-pig liver	Reduction after		
		2 hours	4 hours	18 hours
1	Slices	++	+++ , crystals	+++ , crystals
2	Brei	++ , sol. +	+++ , crystals, sol. ++	+++ , crystals, sol. +++
3	Brei + cyanide (1/400 <i>M</i>)	++	+++ , crystals	+++ , crystals
4	Washed brei	No	No	±, sol. no
5	Washed brei + washings	++ , sol. ++	+++ , sol. +++	+++ , crystals, sol. +++
6	Washings	Sol. ++	Sol. +++	Sol. +++ , many crystals
7	Washed brei + heated washings, filtered	+	+	++ , a few isolated crystals
8	Washings of heated brei	No	No	Sol. +
9	Ammonium sulphate precipitate of washings	No	No	Sol. +
10	Ammonium sulphate precipitate of washings + washed brei	No	No	++ , sol. +
11	Ammonium sulphate precipitate of washings + heated washings filtered	Sol. +	Sol. ++	Sol. +++ , crystals
12	Heated brei	+++ , sol. no	+++ , sol. no	+, sol. +
13	Washed brei heated	+++ , sol. no	—	+, sol. no
14	Heated brei washed	+++ , sol. no	+++ , sol. no	+++ , sol. no
15	Heated washings, unfiltered	+++ , sol. no	+++ , sol. no	+++ , sol. +

It was surprising to find that heated brei (Exp. 12), almost at once and much more quickly than unheated brei, assumes an intense yellow colour with biliverdin solution. This yellow coloration was limited to the tissue, and crystals of

bilirubin were never observed. It was also obtained with heated washed brei (Exp. 13) which before the heating had not reduced biliverdin, or with heated brei extracted with boiling water (Exp. 14). It is thus undoubtedly an artificial effect caused by the heating. The same phenomenon was observed in the coagulated proteins of heated washings (Exp. 15) and a comparison with Exp. 7 shows that it is linked with the presence of the heat-coagulum, since it does not occur when this has previously been removed by filtration. This reduction is probably caused by SH-groups in the denatured proteins. In spite of the strong yellow coloration of the coagulated proteins the degree of reduction is not great. Often the yellow colour disappears again after a few hours.

It is noteworthy that a slight reduction was still observed in the solution in Exps. 8, 12, 15 where the enzymes had been destroyed by heating but where the water-extractable matter of the tissue was present. It will be seen below that this reduction is probably caused by ascorbic acid.

Reduction of biliverdin by dehydrogenase systems.

Reduction by washed liver brei with different substrates. The experiments described make it probable that the greater part of the reduction of biliverdin is due to the action of dehydrogenase systems using biliverdin as hydrogen acceptor. We have therefore investigated several dehydrogenase systems (known to be present in the liver) for their activity with biliverdin. The first experiments were carried out with washed liver brei to which substrates were added. After what has been said of the water-extractability of the liver dehydrogenases acting on biliverdin, it will not be surprising that the results vary a good deal. Prolonged washing leads to a brei which does not reduce biliverdin even with substrate added since the dehydrogenases have been washed away. On the other hand incomplete washing leaves substrates in the brei sufficient for a reduction of biliverdin without further addition. Substrates are however washed out more readily than the enzymes, and it was thus possible to obtain preparations of washed brei with which the influence of substrate addition could be investigated. These experiments were considered necessary since it was doubtful whether all the enzymes present in the liver could be extracted and, if so, whether they were still active after ammonium sulphate precipitation and dialysis in the "liver enzymes".

Table VIII shows that the washed brei of guinea-pig liver 78 answered the above requirements, whereas liver 44 was evidently extracted too much and liver 92 not sufficiently. Brei 44 (Exp. 1) was completely inactive and remained so even after addition of glucose and cozymase (Exp. 2). Brei 78 showed a very small activity (Exp. 3) which was somewhat increased by the addition of glucose and cozymase. There was also a distinct increase of the rate of reduction by addition of glucose to a mixture of washed brei and boiled filtered washings. In this case we found a considerable reduction without glucose (Exp. 5), since the washings contained other substrates, but the reduction was still accelerated by addition of glucose (Exp. 6), coenzyme being present in the washings. Of other substrates lactate (Exp. 7), formate (Exp. 10) and citrate (Exp. 9) were very efficient, but succinate only slightly so. Of non-enzymic reducers ascorbic acid and glutathione were investigated. The latter showed no reducing activity, but ascorbic acid was clearly active, although not as rapidly as some dehydrogenase systems, particularly lactic dehydrogenase.

The results with brei 92 are less conclusive since the strong reduction without substrate (Exp. 13) showed that the brei was not washed free from substrates. Alcohol, lactate, ascorbic acid and citrate increased somewhat the speed of

Table VIII. *Reduction of biliverdin by washed liver brei with different substrates.*

3 mg. biliverdin in 5 ml. Ringer-bicarbonate. 0.5 g. washed liver brei. 37°, toluene. Exps. 1-19 in test-tubes under liquid paraffin. Exps. 20-23 in open 50 ml. Erlenmeyer flasks.

Exp. No.	Substrate	Reduction after	
		2 hours	16 hours
1	Guinea-pig 44	—	—
2	„ 44	No	No
	0.15 <i>M</i> glucose + 25 units cozymase	No	No
3	„ 78	—	+
4	„ 78	+	++
	0.15 <i>M</i> glucose + 25 units cozymase	+	++
5	„ 78	Boiled washings	++ , crystals
6	„ 78	Boiled washings + 0.15 <i>M</i> glucose	++ , crystals
7	„ 78	0.02 <i>M</i> lactate	++ , crystals
8	„ 78	0.02 <i>M</i> succinate	+
9	„ 78	0.01 <i>M</i> citrate	++ , crystals
10	„ 78	0.04 <i>M</i> formate	++ , crystals
11	„ 78	0.06 <i>M</i> glutathione	±
12	„ 78	0.01 <i>M</i> ascorbic acid	++ , crystals
13	„ 92	—	++ , crystals
14	„ 92	0.02 <i>M</i> lactate	+++ , many crystals
15	„ 92	0.3 <i>M</i> alcohol	+++ , crystals
16	„ 92	0.02 <i>M</i> succinate	++ , crystals
17	„ 92	0.01 <i>M</i> citrate	++ , crystals
18	„ 92	0.04 <i>M</i> formate	++ , crystals
19	„ 92	0.01 <i>M</i> ascorbic acid	+++ , crystals
20	Fowl	6 units cozymase	No
21	„	6 units cozymase + 0.03 <i>M</i> glucose	±
22	Cat	6 units cozymase	±
23	„	6 units cozymase + 0.03 <i>M</i> glucose	++

reduction, and the first three caused a more perfect reduction after 16 hours' incubation (Exps. 13-19).

A few aerobic experiments with washed fowl's and cat's liver brei with and without glucose and with cozymase present show that there is a slight increase of the rate of reduction by glucose in both, somewhat more marked in cat's liver than in fowl's.

From these experiments it appears that a number of dehydrogenase systems can reduce biliverdin. Lactate, alcohol, formate and citrate are the best hydrogen donors under the conditions of the experiment, less so glucose and succinate. Of non-enzymic reducers ascorbic acid is active, but slower than the dehydrogenase systems.

The "liver enzymes".

Before studying the effect of the liver enzyme mixture on the reduction of biliverdin its action on methylene blue was investigated. In this way the presence of the different dehydrogenases in the preparation was established and a comparison between the hydrogen acceptors, biliverdin and methylene blue was made possible.

As is shown in Table IX, the preparation contains a variety of different dehydrogenases. The time necessary for reduction of 1 ml. methylene blue (1:5000) was determined. For comparison of these experiments with the biliverdin experiments it is necessary to keep in mind that this amount of methylene blue corresponds to 0.4 mg. of biliverdin, whereas the amount actually

Table IX. *Reduction of methylene blue by dehydrogenase systems in the "liver enzyme mixture".*

1 ml. methylene blue (1 : 5000), 3 ml. enzyme. 0.2 *M* phosphate buffer, pH 7.6. Total volume 7 ml. In vacuum tubes at a low pressure of nitrogen. In experiments with cozymase 12 units of cozymase. Reduction time in min.

Without substrate	No (> 480)	Washings	88
Cozymase without enzyme	No (> 480)	Distillate of washings	195
Cozymase	19	Succinate 0.01 <i>M</i>	24
Glucose 0.01 <i>M</i>	165	Citrate 0.007 <i>M</i>	64
Glucose 0.01 <i>M</i> + cozymase	23	Formaldehyde 0.02 <i>M</i>	28
Lactate 0.01 <i>M</i>	90	Acetaldehyde 0.02 <i>M</i>	1
Lactate 0.01 <i>M</i> + cozymase	13	Salicylaldehyde 0.002 <i>M</i>	1½
Alcohol 0.01 <i>M</i>	135	Xanthine 0.0003 <i>M</i>	7
Alcohol 0.01 <i>M</i> + cozymase	16		

used in the biliverdin experiments was 3 mg., i.e. 7.5 times as much. A smaller amount of biliverdin would not have allowed quantitative determinations. From this it can be understood why the reduction times in all the biliverdin experiments described below are much longer than the decoloration times of methylene blue. By some experiments with an amount of methylene blue corresponding to 3 mg. biliverdin (1.5 ml. methylene blue 1 : 1000) we verified that the reduction time was roughly 7.5 times as long as in experiments with 1 ml. 1 : 5000 methylene blue.

The experiments were carried out in vacuum tubes at a low pressure of nitrogen. The concentration of the substrate was in general 0.01 *M*, with the exception of citrate, and of aldehydes and xanthine of which optimum concentrations were employed. Since the substrate concentration is not optimum in all cases, an exact comparison of the effects of the different enzyme systems is not possible nor was this intended. The data suffice to establish the presence of different dehydrogenases and to demonstrate roughly their strengths in the preparation.

Some dehydrogenase systems require the presence of cozymase; this was prepared from bottom yeast (supplied by Tooth and Co. Ltd., Kent Brewery, Sydney), according to Myrbäck [1931], the purification being carried as far as the recovery from the mercury salt by H_2S . Cozymase when added to the "liver enzymes" produces a reduction of methylene blue without addition of substrates, which somewhat disturbs the study of those enzyme systems for which cozymase is necessary. Neither cozymase alone nor the "liver enzymes" alone reduced methylene blue. Since the velocity of the reaction in the presence of cozymase varies with the "liver enzyme" used, it is probably due to the presence of small amounts of a substrate in the enzyme preparation which requires activation by cozymase. The most likely explanation is that this is desmo-glycogen although its presence could not be demonstrated by other means. Urban [1936] has recently observed that liver protein from cat's liver, prepared in a way similar to that yielding the enzyme preparation, contains small amounts of an unidentified carbohydrate.

Glucose without cozymase causes very slow reduction and the reduction in presence of cozymase is not accelerated by glucose. Lactate however which, without cozymase, causes a reduction in about half the time of glucose, accelerates the reduction in presence of cozymase (although the lactate concentration was much less than optimum), and so does alcohol. If the reduction time due to the endogenous substrate-cozymase system is subtracted, a theoretical reduction time of 43 min. for lactate in presence of cozymase is obtained instead of 90 min. without cozymase. Acetaldehyde and also salicylaldehyde reduce very

quickly and, since heating of the "liver enzyme" destroys this effect, it is due to the action of an enzyme. Xanthine reduces also, but distinctly less. The "liver enzyme" preparation also contains succinic dehydrogenase and (somewhat less) citric dehydrogenase (in the latter case the substrate concentration was nearly optimum). It reduces methylene blue not only with the filtrate from the ammonium sulphate precipitation of the enzymes, but also with a distillate of this filtrate which gives slight aldehyde reactions. The ratio of the velocities with filtrate and with the distillate is however no correct indication of the role which the oxidation of volatile aldehydes plays in the liver since the distillate was obtained from many times the amount of filtrate.

*Reduction of biliverdin by dehydrogenase systems in the
"liver enzyme" preparation.*

The presence of a number of dehydrogenases in the "liver enzyme" preparation having thus been established, we proceeded to examine their effects on biliverdin reduction and the results are shown in Table X.

Cozymase along with enzyme but without substrate has little effect on the reduction of biliverdin, and no difference was found in experiments with different substrates with or without cozymase, which seems rather remarkable.

Glucose, in the presence of the enzyme preparation, reduced biliverdin very little (Exps. 3 and 4, Table X).

Table X. *Reduction of biliverdin by dehydrogenase systems in the
"liver enzyme" preparation.*

3 mg. biliverdin in 0.1 ml. 0.2 *N* alkali. 3 ml. enzyme in phosphate buffer *pH* 7.6. Total volume 7 ml. In test-tubes under liquid paraffin. Toluene. In experiments with cozymase 12 units of cozymase were added.

Exp. No.	Substrate	Guinea- pig 55 16 hours	Guinea-pig 303			Guinea-pig 31			Rabbit 16 hours
			1 hour	2 hours	16 hours	A	B	C	
1	—	No	No	No	+	±	±	±	No
2	Cozymase	No	—	—	—	—	—	—	—
3	0.01 <i>M</i> glucose	+	—	—	—	—	—	—	—
4	0.01 <i>M</i> glucose + cozymase	+	—	—	—	—	—	—	—
5	0.02 <i>M</i> lactate	+, crystals	—	—	—	—	—	—	—
6	0.02 <i>M</i> lactate + cozymase	+, crystals	+	++	+++, crystals	+, crystals	+, crystals	+, crystals	+
7	0.3 <i>M</i> alcohol	+, crystals	±	—	+++, many crystals	—	—	—	—
8	0.3 <i>M</i> alcohol + cozymase	+, crystals	—	—	—	+, crystals	+, crystals	±	+
9	0.02 <i>M</i> succinate	—	No	+	++	+	+	±	No
10	0.01 <i>M</i> citrate	—	No	+	++	+	+	±	—
11	0.04 <i>M</i> formate	—	—	—	—	+	+	+	—
12	0.005 <i>M</i> xanthine	No	—	—	—	±	±	±	—
13	0.2 <i>M</i> acetalde- hyde	—	++	+++, many crystals	+++, many crystals	+++, crystals	+++, crystals	+++	+++, crystals
14	0.005 <i>M</i> salicyl- aldehyde	+++, many crystals	—	—	—	—	—	—	—

A. Extract with water
B. Extract with secondary phosphate
C. Extract with primary phosphate } Reduction time 16 hours.

Lactate and alcohol (the latter was used at rather high concentrations) are excellent hydrogen donors for biliverdin; the fastest reducers, however, are aldehydes. Neither acetaldehyde nor salicylaldehyde reacts with biliverdin alone. It was suspected that the xanthine oxidase of the liver catalysed this reaction; xanthine however had a very slight effect, if any. Succinate, citrate and formate also gave a slight reduction of biliverdin.

On the whole the results agree with those obtained with washed brei. With "liver enzyme" citrate and formate appeared to be less active than with the brei; in the case of citrate this was certainly due to the small concentration of citric dehydrogenase in the enzyme preparation. Lactate and alcohol produced a good reduction with the enzyme preparation, although the former was used in sub-optimum concentration and the enzyme preparation did not contain much alcohol dehydrogenase.

It does not make a great difference whether water, secondary phosphate or primary phosphate is used for the extraction of the enzymes. Extracts with primary phosphate contain less alcohol, succinic and citric dehydrogenases than extracts with water or secondary phosphate, to judge from the biliverdin reduction experiments.

Roughly quantitative experiments with "liver enzyme" and other dehydrogenase preparations.

Technique and estimation. 3 mg. of biliverdin were incubated with the enzyme and some toluene in 0.2 *M* phosphate buffer, pH 7.6, in vacuum tubes at low pressure nitrogen.

After incubation at 37° biliverdin and bilirubin were removed from the proteins as quantitatively as possible and separated from each other in the following way.

The mixture was made alkaline with a few ml. 0.2 *N* NaOH and 10 ml. of methyl alcohol were added. This solution was poured into a separating funnel containing 10 ml. of ether and a slight excess of glacial acetic acid. It was shaken at once (not too vigorously!) and several times extracted with ether until no more pigment was removed. Proteins were precipitated in the aqueous layer which was filtered (filtrate C). The protein precipitate on the filter was boiled out successively with methyl alcohol and with chloroform. In most cases these extracts did not contain any appreciable amount of pigment. If they did the methyl alcoholic extracts were again distributed between ether and dilute acetic acid, and from the chloroform extracts the pigments were extracted with 0.2 *N* NaOH, and transferred to ether in the way described above. The united ethereal extracts were washed with water, and biliverdin was isolated by two extractions with a few ml. of 1% HCl followed by one extraction with 5% HCl (A). From the remaining ether solution, after washing with water, bilirubin was extracted with a few ml. of 0.2 *N* NaOH (B).

Biliverdin was determined in A by adding methyl alcohol up to 50 ml. and comparing this solution colorimetrically with a biliverdin standard in methyl alcoholic HCl.

In B bilirubin was determined by the Van den Bergh reaction after addition of alcohol and a few drops of glacial acetic acid. Biliverdin (and also meso-biliverdin) does not react with diazobenzenesulphonic acid in acid solution.

The aqueous solution C was often colourless, but sometimes contained a considerable amount of bilirubin with the characteristic properties of "direct bilirubin". The pigment was not extracted from the aqueous solution by chloroform and gave the direct Van den Bergh test, with a somewhat more

bluish violet colour which, however, turned to the typical red-violet when alcohol was added. Bilirubin in C was determined by the Van den Bergh reaction, alcohol being added after the colour had been developed with the diazo-reagent.

As standard we used cobaltous sulphate for determinations of weak solutions of the azo-dye. Stronger solutions were matched more satisfactorily against an acid methyl red solution. Both solutions were standardized with pure crystalline bilirubin. The cobaltous sulphate standard was equivalent to a bilirubin concentration of 0.5 mg. in 100 ml., the methyl red standard to a concentration of 2 mg. in 100 ml.

The sum of A, B and C was often 70 % or more of the amount of biliverdin used. In many cases however the protein precipitate after extraction still contained as much as 70–80 % of the total pigment. These precipitates were always yellow and did not contain an appreciable amount of biliverdin, which was much less adsorbed on the proteins than bilirubin. The pigment contained in the precipitate therefore was assumed to be bilirubin.

The rate of reduction is estimated with a maximum error of about $\pm 10\%$ of the original biliverdin.

Glucose and biliverdin. In a number of experiments with liver slices of the guinea-pig and the rat no accelerating action of glucose on the aerobic reduction of biliverdin was found, but in other experiments a distinct acceleration was observed like that reported above with liver slices of the starved guinea-pig (Exp. 16, Table I). Glucose has also been found to accelerate the anaerobic reduction in guinea-pig liver slices (Exp. 2, Table V), in washed brei of the fowl (Exp. 21, Table VIII), and the cat (Exp. 23, Table VIII), and somewhat with the enzyme preparation of guinea-pig's liver (Exps. 3, 4 and 5, Table X). With washed brei and with enzyme mixture the acceleration was however much less marked than in those experiments with slices where it had been observed. These observations and the fact that lactic acid is a suitable hydrogen donor for biliverdin make it probable that glucose is first glycolysed and the lactic acid thus formed acts as hydrogen donor.

The glucose dehydrogenase of the liver had also to be taken into account. This enzyme was prepared from horse liver according to the procedure of Harrison [1933]. The amounts of enzyme and coenzyme used corresponded to 0.8 g. of acetone-liver. In another experiment cozymase was added.

Table XI. *Reduction of biliverdin and of methylene blue by the glucose dehydrogenase system of horse liver.*

Exp. No. ...	1	2	3	4
Enzyme (ml.)	0.4	0.4	0.4	0.4
Coenzyme (ml.)	0.5	—	0.5	—
Cozymase (12 units) (ml.)	—	1.0	—	1.0
Glucose 2 M (ml.)	0.2	0.2	0.2	0.2
Methylene blue 1 : 5000 (ml.)	—	—	0.4	0.4
Biliverdin 3 mg. (ml.)	0.2	0.2	—	—
Phosphate buffer pH 7.6 (ml.)	0.7	0.2	0.7	0.2
Reduction	0.6 mg. in 50 hours	0.6 mg. in 50 hours	60 min.	15 min.

Table XI shows that the enzyme preparation was somewhat less active to methylene blue than that of Harrison. The reduction of biliverdin which is caused is so small and slow that it remains doubtful whether the enzyme acts at all with biliverdin. This action is certainly much slower than that with

methylene blue, although the amount of methylene blue used was 20 times smaller than that of biliverdin.

The amount of acetone-liver from which enzyme and coenzyme had been obtained, corresponded to at least 5 times the amount of fresh liver tissue used for the qualitative anaerobic experiments in which almost complete reduction had been obtained in 4 hours. Unless the greater part of the enzyme present in the fresh liver was destroyed by the preparation, we conclude that glucose dehydrogenase cannot play a role in biliverdin reduction in the liver.

Lactate and biliverdin. It has been shown in experiments with washed liver brei (Exps. 7 and 14, Table VIII), and with "liver enzyme" (Exps. 6 and 7, Table X), that lactate is effective as hydrogen donator for biliverdin. Table XII shows that the rate of reduction of biliverdin with lactate in presence of "liver enzyme" is much greater than that caused by glucose dehydrogenase.

The amount of "liver enzyme" was the same as used for the qualitative experiments and corresponded to about 0.4 g. liver.

Table XII. *Reduction of biliverdin and of methylene blue by lactic dehydrogenase of guinea-pig liver.*

Exp. No. ...	1	2	3	4	5	6
Enzyme (ml.)	3	—	3	3	3	3
Heated enzyme (ml.)	—	3	—	—	—	—
Cozymase, 12 units (ml.)	—	—	1	1	1	1
Lactate 1% (ml.)	0.45	0.45	0.45	—	0.45	—
Methylene blue 1 : 5000 (ml.)	—	—	—	—	1	1
Biliverdin 3 mg. (ml.)	1	1	1	1	—	—
Phosphate buffer pH 7.6 (ml.)	2.55	2.55	1.55	2	1.55	2
Reduction	1.5 mg. in 16 hours	No	1.5 mg. in 16 hours	No	30 min.	38 min.

Cozymase did not cause a reduction of biliverdin whether without or with enzyme (Exp. 4), and did not accelerate the rate of biliverdin reduction with lactic acid (cf. Exps. 3 and 1). The enzymic nature of the reduction follows from the fact that heating of the enzyme destroys its activity (Exp. 2). The rate of methylene blue reduction cannot be established quite clearly since cozymase produces a considerable speed of reduction of methylene blue by the "liver enzyme" without substrate. From the difference of the reduction times with and without lactate one can estimate that the lactic dehydrogenase system brings about the reduction of an amount of methylene blue equivalent to 3 mg. biliverdin in approximately 18 hours. This calculation is very inaccurate, but it allows the statement that lactic dehydrogenase does not react much more slowly with biliverdin than with methylene blue, at least if low concentrations of lactate are used. The lactic acid dehydrogenase system probably accounts for a considerable part of the biliverdin reduction in the liver.

It was considered interesting to investigate whether lactic acid dehydrogenase from other sources would be equally capable of using biliverdin as hydrogen acceptor. Table XIII shows that an enzyme prepared from zymin of bottom

Table XIII. *Reduction of biliverdin and of methylene blue by yeast lactic acid dehydrogenase.*

Enzyme ml.	Lactate 0.5 N ml.	Methylene blue 1 : 5000 ml.	Biliverdin 3 mg. ml.	Phosphate buffer pH 7.6 ml.	Reduction
3	1	1.5	—	1.5	15 min.
3	1	—	1	2	0.6 mg. in 48 hours

yeast according to Bernheim [1928] was very powerful in reducing methylene blue even without cozymase but was almost inactive towards biliverdin, although an optimum concentration of lactate was used.

Alcohol and biliverdin. Alcohol has been shown to increase the rate of biliverdin reduction by washed liver brei (Exp. 15, Table VIII), and by "liver enzyme" (Exps. 8 and 9, Table X). Table XIV shows that liver enzyme mixture with alcohol as substrate reduces biliverdin rapidly. As in the case of lactic acid dehydrogenase there is no great difference between methylene blue and biliverdin as hydrogen acceptors, but the same difficulties prevent an exact determination of the rate of reduction of methylene blue. The reduction time of an amount of methylene blue equivalent to 3 mg. biliverdin can be roughly estimated to be 18 hours.

Table XIV. *Reduction of biliverdin and of methylene blue by alcohol dehydrogenase from guinea-pig liver.*

Exp. No. ...	1	2	3	4	5
Enzyme (ml.)	3	3	3	3	—
Heated enzyme (ml.)	—	—	—	—	3
Cozymase, 12 units (ml.)	1	1	—	1	1
Alcohol 80 mg. (ml.)	0.1	—	0.1	0.1	0.1
Methylene blue 1 : 5000 (ml.)	1	1	—	—	—
Biliverdin 3 mg. (ml.)	—	—	1	1	1
Phosphate buffer pH 7.6 (ml.)	1.9	2.0	2.9	1.9	1.9
Reduction	30 min.	38 min.	2.4 mg. in 16 hours	2.4 mg. in 16 hours	No

Succinate and biliverdin. Succinate (0.02 *M*) has been shown above to accelerate biliverdin reduction in washed liver very little if at all (Exp. 16, Table VIII), somewhat more in some experiments with "liver enzyme" (Exp. 10, Table X). The experiment set up to estimate the amount of bilirubin formed by succinate and "liver enzyme" failed however to show any reduction in 18 hours, beyond the small reduction given by the enzyme without substrate.

Succinic dehydrogenase of bottom yeast was prepared from zymon according to Bernheim [1928]. Table XV shows that it caused biliverdin reduction, being a stronger succinic dehydrogenase than the "liver enzyme" (the reduction time of 7.5 times the amount of methylene blue was only twice as long). It is probable that the failure to obtain reduction with the "liver enzyme" was due to a destruction of the rather sensitive enzyme during the long period necessary for reduction of 3 mg. biliverdin. We found also that 1.5 ml. methylene blue (1:1000) could not be completely decolorized by "liver enzyme" in presence of succinate. Even so, the succinic dehydrogenase system reacts much more slowly with biliverdin than it does with methylene blue.

Table XV. *Reduction of biliverdin and of methylene blue by succinate in presence of "liver enzyme" and of succinic dehydrogenase from bottom yeast.*

Exp. No. ...	1	2	3	4	5	6
Liver enzyme (ml.)	3	3	3	—	—	—
Yeast enzyme (ml.)	—	—	—	3	3	3 (heated)
Succinate 1% (ml.)	2	2	2	2	2	2
Methylene blue 1 : 5000 (ml.)	1	—	—	—	—	—
Methylene blue 1 : 1000 (ml.)	—	1.5	—	1.5	—	1.5
Biliverdin 3 mg. (ml.)	—	—	1	—	1	—
Phosphate buffer pH 7.6 (ml.)	1	0.5	1	0.5	1	0.5
Reduction	24 min.	>24 hours	Trace in 18 hours	46 min.	1.5 mg. in 18 hours	Trace in 18 hours

Citric acid and biliverdin. Citrate has been found active in reducing biliverdin in both washed liver brei (Exps. 9 and 17, Table VIII) and "liver enzyme" (Exp. 11, Table X). The quantitative experiment (Exp. 2, Table XVI) confirms that the citric dehydrogenase system is little inferior to lactic dehydrogenase in biliverdin reduction. The "liver enzyme" citric dehydrogenase is only slightly more active to methylene blue (Exp. 1) than to biliverdin. Citric dehydrogenase was also prepared from horse acetone-liver according to Bernheim [1928] with removal of haemoglobin. Its activity was slightly less than that of the guinea-pig "liver enzyme" (Exps. 3 and 4). It did not reduce methylene blue without substrate (Exp. 5) and it was destroyed by heating (Exp. 6).

Table XVI. *Reduction of biliverdin and of methylene blue by citric dehydrogenases from guinea-pig liver enzyme and from horse acetone liver.*

Exp. No. ...	1	2	3	4	5	6
Guinea-pig liver enzyme (ml.)	3	3	—	—	—	—
Horse liver enzyme (ml.)	—	—	3	3	3	3 (heated)
Citrate 1% (ml.)	1	1	1	1	1	1
Methylene blue 1:1000 (ml.)	1.5	—	1.5	—	1.5	1.5
Biliverdin 3 mg. (ml.)	—	1	—	1	—	—
Phosphate buffer pH 7.6 (ml.)	1.5	2	1.5	2	1.5	1.5
Reduction	19 hours	1 mg. in 16 hours	27 hours	2.1 mg. in 48 hours	No	No

Aldehydes and xanthine as hydrogen donators for biliverdin. Acetaldehyde has been found to be the most efficient hydrogen donator for biliverdin in experiments with liver enzyme preparation (Exp. 14, Table X), and salicylaldehyde (Exp. 15, Table X) to be similarly active. These experiments were confirmed by roughly quantitative experiments (Exps. 1 and 5, Table XVII). Acetaldehyde reduces biliverdin rapidly in presence of "liver enzyme" preparation (Exp. 1), whereas the enzyme without substrate (Exp. 2) reduces very little. Salicylaldehyde (Exp. 5) is hardly less active; increasing its concentration above 0.004 *M* makes no difference. Neither aldehyde alone causes any reduction (cf. Exps. 4 and 8, Table XIX).

It was to be expected that the xanthine oxidase of the liver would reduce biliverdin and it has been shown above (Table IX) that the "liver enzyme" preparation from guinea-pig's liver contains xanthine oxidase. Experiments with xanthine oxidase from milk seemed to support the view that the reduction of biliverdin by aldehydes was caused by this enzyme. Two kinds of preparations were employed: the caseinogen preparation of Dixon & Thurlow [1924] and a preparation according to an unpublished method of Dixon and Lemberg, which we shall call here "globulin preparation". The latter is obtained in the following way. Milk is saturated with sodium chloride and the caseinogen-fat precipitate is removed by filtration. From the filtrate the enzyme is now precipitated with ammonium sulphate. The precipitate is dissolved in water, reprecipitated by half-saturation with ammonium sulphate and dissolved in 0.2 *M* phosphate buffer pH 7.6. The caseinogen preparation was employed as solution in the same buffer. These enzymes caused a strong reduction of biliverdin by acetaldehyde (Exps. 1 and 5, Table XVIII) and by salicylaldehyde (Exp. 9, Table XVIII). No reduction was observed with formaldehyde.

Xanthine did not however cause a biliverdin reduction either with "liver enzyme" of the guinea-pig (Exps. 13, Table X; Exp. 7, Table XVII) or with xanthine oxidase from milk (Exp. 11, Table XVIII). For complete reduction of the amount of biliverdin used for these experiments it was necessary to use a

xanthine concentration above that found optimum by Dixon & Thurlow [1924]. The possibility cannot yet be excluded that the access of biliverdin to the enzyme surface is prevented by excess xanthine more effectively than that of methylene blue, and that lower concentrations of xanthine would cause reduction of biliverdin. We have tried to verify this by using 0.0004 *M* xanthine which should still suffice to reduce 1.5 mg. of the 3 mg. of biliverdin added. If there is a reduction—in some instances the reduction with xanthine was slightly greater than in the control—it is certainly very small.

It can be shown indeed that the enzyme preparations activate aldehydes for the reaction with biliverdin in another way. A large part of the catalytic action of the "enzyme mixture" and of milk xanthine oxidase on acetaldehyde is not due to an enzymic action at all, since an enzyme solution boiled for 3 min. proved no less active (Exp. 3, Table XVII; Exps. 3 and 7, Table XVIII). Heated liver enzyme alone (Exp. 4, Table XVII) reduces biliverdin very little, heated milk enzymes (Exps. 4 and 8, Table XVIII) slightly more. Salicylaldehyde differs from acetaldehyde in being much less activated by "heated enzymes" (Exp. 6, Table XVII; Exp. 10, Table XVIII).

Table XVII. *Reduction of biliverdin by "liver enzyme" of the guinea-pig with aldehydes and xanthine.*

Exp. No. ...	1	2	3	4	5	6	7
Enzyme (ml.)	3	3	3	3	3	3	3
			heated	heated		heated	
Acetaldehyde 5% (ml.)	1	—	1	—	—	—	—
Salicylaldehyde* (ml.)	—	—	—	—	1	1	—
Xanthine 4 mg. (ml.)	—	—	—	—	—	—	1
Biliverdin 3 mg. (ml.)	1	1	1	1	1	1	1
Phosphate buffer pH 7.6 (ml.)	2	3	2	3	2	2	2
Reduction in 16 hours	2.7 mg.	<0.2 mg.	2.1 mg.	<0.2 mg.	2.7 mg.	0.6 mg.	<0.2 mg.

* 4 and 50 mg. of salicylaldehyde gave the same result.

Table XVIII. *Reduction of biliverdin by aldehydes and xanthine in presence of xanthine oxidase from milk.*

Exp. No. ...	1	2	3	4	5	6	7	8	9	10	11
Caseinogen prep. 200 mg. (ml.)	3	3	—	—	—	—	—	—	3	—	3
Caseinogen heated (ml.)	—	—	3	3	—	—	—	—	—	3	—
Globulin prep. (ml.)	—	—	—	—	3	3	—	—	—	—	—
Globulin prep. heated (ml.)	—	—	—	—	—	—	3	3	—	—	—
Acetaldehyde 5% (ml.)	1	—	1	—	1	—	1	—	—	—	—
Salicylaldehyde 4 mg. (ml.)	—	—	—	—	—	—	—	—	1	1	—
Xanthine 1 mg. (ml.)	—	—	—	—	—	—	—	—	—	—	1
Biliverdin 3 mg. (ml.)	1	1	1	1	1	1	1	1	1	1	1
Phosphate buffer pH 7.6 (ml.)	2	3	2	3	2	3	2	3	2	2	2
Reduction in 16 hours	3	<0.2	3	0.3	1.5	<0.2	1.5	0.3	2.1	0.6	<0.2
	mg.	mg.	mg.*	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.

* In 5 hours less biliverdin was reduced in Exp. 3 than in Exp. 1.

With caseinogen preparation the reduction by the unheated preparation (Exp. 1) was faster (after 5 hours) than that by the heated preparation (Exp. 3); after 17 hours, reduction was complete in both cases. With the globulin preparation no difference was observed between heated and unheated enzymes (Exps. 5 and 7). Without aldehyde the heated preparations caused a slight reduction, somewhat more than heated liver enzyme.

These results prove the presence of a heat-stable catalyst in the preparations from liver and milk activating the reduction of biliverdin with acetaldehyde and

to a minor degree with salicylaldehyde. The experiments of Table XIX show that this catalyst is nonspecific.

Table XIX. *Reduction of biliverdin by aldehydes in presence of amino-compounds.*

Exp. No. ...	1	2	3	4	5	6	7	8	9	10
Egg albumin 200 mg. (ml.)	3	—	—	—	3	—	—	—	3	—
Egg albumin 200 mg. heated (ml.)	—	3	—	—	—	3	—	—	—	3
Glycine 200 mg. (ml.)	—	—	3	—	—	—	3	—	—	—
Acetaldehyde 5% (ml.)	1	1	1	1	—	—	—	—	—	—
Salicylaldehyde 4 mg. (ml.)	—	—	—	—	1	1	1	1	—	—
Biliverdin 3 mg. (ml.)	1	1	1	1	1	1	1	1	1	1
Phosphate buffer pH 7.6 (ml.)	2	2	2	5	2	2	2	5	3	3
Reduction in 18 hours	2.1	3.0	3.0	No	No	No	0.9	No	No	<0.2
	mg.	mg.	mg.				mg.			mg.

Egg albumin (Exp. 1), heat-coagulated egg albumin (Exp. 2) and glycine (Exp. 3) are able to activate acetaldehyde. Salicylaldehyde (Exps. 5–7) is also activated by glycine, but distinctly less so, and not by egg albumin. Exps. 9 and 10 are controls with egg albumin and heat-coagulated egg albumin showing that there is no reduction, or only a very slight one, without substrate. Xanthine, as would be expected, is not activated by amino-compounds. Exps. 4 and 8 show that the aldehydes need activation to react with biliverdin.

That amino-compounds activate aldehydes also towards methylene blue has been found by Haehn [1926]. This system has been studied in detail by Lieben & Getreuer [1932; 1934]; it probably involves the formation of Schiff's bases. The reduction of methylene blue by aldehydes in presence of amino-compounds however requires a higher temperature (75°) than that of biliverdin and at 37° its velocity is negligible.

These observations explain the rapid reduction of biliverdin by acetaldehyde in presence of liver enzyme or of milk xanthine oxidase, but not sufficiently the reduction by salicylaldehyde. This aldehyde is not much activated by the heat-stable system, and the observations on biliverdin reduction by xanthine do not make it probable that the strong reducing action of salicylaldehyde on biliverdin can be explained by the action of xanthine oxidase.

Since however biliverdin reduction is complicated by the action of the heat-stable system and its rate cannot be determined very accurately, experiments with methylene blue and guinea-pig liver enzyme were carried out. We have compared the velocities of methylene blue decoloration by aldehydes and xanthine in presence of liver and milk enzyme preparations, and have, in contrast to Booth [1935], arrived at the conclusion that the activation of aldehydes in the liver—at least as far as guinea-pig's and rabbit's liver is concerned—cannot be satisfactorily explained by the assumption that aldehydes are activated by the one enzyme, xanthine oxidase, alone. These experiments will be described in a second paper. They make it probable that the activation of salicylaldehyde towards biliverdin is due to the action of an aldehyde dehydrogenase different from xanthine oxidase.

This aldehyde dehydrogenase works with acetaldehyde also. Against this one might object that no considerable difference has been found between the actions of unheated and heated "liver enzymes" with acetaldehyde (cf. Exps. 1 and 3, Table XVII). A comparison of the experiments with unheated and heated egg albumin (Exps. 1 and 2, Table XIX), however, shows that heat-coagulation increases the efficacy of the proteins so that the activation due to the heat-labile enzyme system is greater than appears from the experiments of Table XVII.

Reduction of biliverdin by non-enzymic reducers. Ascorbic acid when added to washed liver brei has been shown above (Exps. 12 and 19, Table VIII) to cause reduction of biliverdin. The roughly quantitative experiment shows, however, that the reduction by vitamin C is not rapid.

3 mg. of biliverdin (dissolved in 0.1 ml. 0.2 *N* NaOH) were incubated at 37° under nitrogen with a solution of 10 mg. pure crystalline ascorbic acid (B.D.H.) in 5 ml. phosphate buffer pH 7.6. After 24 hours the solution was green-brown and 0.9 mg. of biliverdin had been reduced.

Crystalline glutathione did not cause a reduction of biliverdin in washed liver brei (Exp. 11, Table VIII), nor was any reduction observed when biliverdin was incubated for 3 days at 37° under nitrogen with a solution of 10 mg. crystalline glutathione in phosphate buffer pH 7.6. With impure ("deliquescent") glutathione a slow and partial reduction was obtained.

Reduction of mesobiliverdin in the liver. Like biliverdin, mesobiliverdin can act as hydrogen acceptor to the dehydrogenase systems of the liver. When 3 mg. of mesobiliverdin were incubated under liquid paraffin with guinea-pig liver slices these became intensely yellow after 1 hour, and after 16 hours the tissue was filled with fine needles of mesobilirubin. The heat-stable aldehyde-amino-compound system also reduces mesobiliverdin. Of 3 mg. mesobiliverdin 1.8 mg. were reduced after 16 hours' incubation with acetaldehyde and heated or unheated "liver enzyme" of the guinea-pig. The mesobilirubin crystallized from chloroform in the typical crystal forms and gave positive Gmelin and Van den Bergh reactions.

DISCUSSION.

The experimental data of this paper supply evidence that biliverdin is reduced to bilirubin in animal tissues, particularly in the liver, spleen, kidney and brain, speedily enough to allow the assumption that biliverdin is the primary product of haemoglobin breakdown, and that bilirubin results by a reduction of biliverdin in the body. These facts alone offer, of course, no positive evidence that biliverdin is the primary bile pigment, but, taken together with the *in vitro* model of haemoglobin breakdown in an earlier paper [Lemberg, 1935], and with chemical considerations, they strengthen the evidence in favour of this hypothesis.

Since the liver of the majority of species reduces biliverdin at a rate which greatly surpasses the slow rate of haemoglobin breakdown (quantitative evidence is offered for human and guinea-pig livers), one can hardly expect to find biliverdin in liver bile, even under conditions of increased haemoglobin breakdown.

The bilirubin excreted from the liver into the bile is the so-called "direct" bilirubin, insoluble in organic solvents and giving a direct Van den Bergh reaction. We have observed that reduction of biliverdin by liver enzymes yields a mixture of "direct" and "indirect" bilirubins. Further study is necessary, particularly in order to show whether the stable combination of bilirubin with liver protein, which probably explains the properties of the direct bilirubin, is linked up with the reduction mechanism, or whether it occurs independently of it.

Reduction of biliverdin was observed in all tissues although less markedly elsewhere than in liver, spleen, kidney and brain. Blood was found to be unable to reduce biliverdin, although it retarded bilirubin oxidation. The reduction of biliverdin must therefore occur before this enters the blood stream. It is nevertheless felt that the extra-hepatic bilirubin pigment formation can be explained in the same way, since the principal sites of bile pigment formation were found

to have a strong reducing action on biliverdin. One might object that in certain conditions (e.g. blood extravasations) considerable bilirubin formation is observed so that the relatively small reducing power, e.g. of muscle tissue, would not account for a sufficiently rapid reduction. The tissues however under these conditions are not comparable with normal tissue examined in slices in aerated solution, and nothing, moreover, is known about the reducing power within the phagocytic cells which are amassed in such haemorrhages and in which the haemoglobin breakdown occurs. One instance where extensive haemorrhages lead to formation of biliverdin and not of bilirubin is known, viz. the formation of uteroverdin in the dog's placenta [Lemberg & Barcroft, 1932], and biliverdin has often been observed side by side with bilirubin in the tissues.

Liver tissue was found to prevent bilirubin oxidation and for this reason the finding of biliverdin in fresh bile, particularly in liver bile, is important evidence. Biliverdin has been found in the bile mainly under conditions in which the storage of substrates (e.g. glycogen) in the liver can be considered as diminished: in the bile of starving animals, or in transfusion experiments with frog's liver. (In our experiments, frog's liver showed a smaller reducing power than mammalian or avian livers.) It has been shown in this paper that the dehydrogenase systems of the liver play an important role in biliverdin reduction. Hence, the impoverishment of the liver with regard to substrates will decrease its reducing power.

The substances capable of producing reduction of biliverdin in the liver are aldehydes, glucose, lactic acid, alcohol, citrate, ascorbic acid and to a less extent succinate. Ascorbic acid does not react rapidly with biliverdin, and some of the dehydrogenase systems reduce it more rapidly than ascorbic acid, so that the reduction of biliverdin cannot be explained by an assumption of a catalytic role of ascorbic acid. Aldehydes are activated to react with biliverdin by proteins as heat-stable catalysts and by an aldehyde dehydrogenase probably different from xanthine oxidase, whereas it is doubtful whether the last-mentioned enzyme can react with biliverdin, since xanthine could not be used as substrate.

The liver dehydrogenases capable of producing a rapid reduction of biliverdin are lactic dehydrogenase, alcohol dehydrogenase, and (somewhat less) citric dehydrogenase. It was interesting to see how the different systems would react with a hydrogen acceptor which, in contradistinction to methylene blue, is physiological, although of limited importance in this role in the body. Biliverdin, moreover, does not appear to form a reversible redox system with bilirubin. We have been unable to observe any reaction either of bilirubin with methylene blue or of biliverdin with leucomethylene blue under nitrogen. All dehydrogenase systems act more quickly with methylene blue, but in some of them (alcohol, citric and lactic dehydrogenases) the difference is less marked than in others (glucose, succinic and aldehyde dehydrogenases), and no action of biliverdin was observed with yeast lactic dehydrogenase or with xanthine oxidase. The heat-stable aldehyde-protein system is the only one found to react more rapidly with biliverdin than with methylene blue.

The study of the action of dehydrogenases on the reduction of biliverdin is, admittedly, still incomplete. Some of the more important dehydrogenases of the liver (e.g. hexosephosphate dehydrogenases, glycerophosphate dehydrogenase) have not yet been investigated. This might be the reason why the action of the established reducing systems appears not quite sufficient to account for the reduction actually observed in the tissues. Of those dehydrogenases found to reduce biliverdin rapidly, only lactic dehydrogenase is supposed to play an important role in the liver, whereas other important enzyme systems, such as

glucose dehydrogenase, succinic dehydrogenase and xanthine oxidase, were not very active or inactive with biliverdin. On the other hand, an exact comparison between the rate of reduction in the tissue and that with the isolated enzyme systems is, of course, impossible for many reasons.

Note by R. Lemberg.

The observations described above show that mesobiliverdin is reduced in the tissues to mesobilirubin, and that it is reduced by the heat-stable aldehyde system like biliverdin.

Siedel [1935] has recently observed that mesobiliverdin if injected subcutaneously disappears more rapidly than mesobilirubin and has assumed for this reason that it plays a physiological role as the "blue colour" of traumatic haemorrhages without, however, offering evidence for this assumption.

Siedel [1935] disputes the identity of the nuclear structure of mesobiliverdin with that of biliverdin, and hence the propriety of using the name "mesobiliverdin" for the substance which Fischer and his school call "glauco bilin". On the former point I have adduced evidence which I consider conclusive [Lemberg, 1934, 1; 1935]. Both compounds contain two atoms of hydrogen less than the corresponding rubins and are obtained from these in the same way; both are obtained from the corresponding haemins in the same way. In the typical properties (formation of salts and ferri chlorides, solubility and basicity, light absorption of the hydrochlorides and behaviour to oxidizing agents) the two compounds show no differences. In the meantime Fischer and his school have admitted that glauco bilin constitutes the green, and not the blue, oxidation stage of mesobilirubin. In his recent paper Siedel has brought experimental evidence against his earlier hypothesis that the presumable structural differences of the tetrapyrrene systems in biliverdin and mesobiliverdin were due to a "keto-enol" isomerism (better called lactim-lactam isomerism). In spite of this and of the principal objections raised against this assumption by Lemberg [1935], Siedel has not yet abandoned this hypothesis. There are slight differences in colour of the crystals in *thin* layers (both biliverdin and mesobiliverdin crystals are "steel-blue") and of the neutral solutions (those of mesobiliverdin being a purer blue than those of biliverdin), but I do not feel that they justify the assumption of a genuine structural difference. They are either caused by the difference in the side-chains, or by their influence on the fine structure which our formulae are too coarse to express. These differences disappear on salt formation. I have never observed a blue colour of mesobiliverdin hydrochloride; in the one case which Siedel perhaps had in mind in making his statement I have proved that the blueness of the colour was due to admixture of mesobiliviolin.

Against the use of the name "mesobiliverdin" Siedel refers to the fact that Fischer has thus designated the compound obtained by autoxidation of mesobilirubin in alkaline solution. He has overlooked the fact that I have isolated this compound and have identified it with "glauco bilin", this being an important reason for preserving the older and well-fitting name. Since the verdins are indicator substances it does not matter to which colour, blue or green, the name applies. "Oocyan" was used for biliverdin, because in the egg shells the colour is predominantly blue, "utero verdin" because in the tissue the colour is green. Mesobiliverdin also gives a green colour to tissue and not a blue one as Siedel assumes.

The use of inappropriate names by Fischer's school makes the understanding of bile pigment chemistry unnecessarily difficult to a wider circle of scientists. A name like "ferrobilin" for the ferri chloride of mesobiliverdin [Fischer *et al.* 1932] is unnecessary and causes mistakes when employed to designate both ferri chloride and ferri bromide as has been done in this paper. The term "mesobiliviolin" is used by Siedel for a compound isomeric with mesobilirubin synthesized by him, without even mentioning the fact that the same name is applied to an oxidation product of mesobilirubin [Lemberg, 1934, 2]. Names like "copromesobiliviolin" are misleading once the prefix "copro" has been chosen to designate the kind of side-chains ("coproporphyrin", "coprobiliverdin"). Since the non-identity of urobilinogen (from both faeces and urine) with mesobilirubinogen (Watson, Lemberg) has been established, and has been confirmed by Fischer, the terms urobilinogen and urobilin should no longer be applied for the artificial products. Nor can I see any reason for replacing my term mesobiliviolin by mesobilirhodin.

Siedel's remarks on the constitution of the phycobilins will be discussed in a forthcoming paper.

SUMMARY.

Under anaerobic conditions biliverdin is reduced to bilirubin by all tissues of the guinea-pig investigated, skin being an exception, and also under aerobic conditions in liver, spleen, kidney and brain. The livers of all animals investigated were found to possess this strong reducing power for biliverdin, frog's liver alone being found distinctly less reducing than others. The significance of these findings, on the hypothesis that biliverdin is the primary bile pigment, is discussed.

Biliverdin and mesobiliverdin are reduced to the corresponding rubins by zinc dust in ammoniacal solution, and mesobiliverdin is also reduced by liver tissue.

The reduction of biliverdin in guinea-pig liver is caused by a number of dehydrogenase systems and by ascorbic acid. Aldehydes, glucose, lactate, alcohol, citrate and formate were found to act as hydrogen donors to biliverdin in the presence of liver enzymes, less so succinate. Glucose dehydrogenase is slightly active, but the greater part of the activity of glucose is due to glycolytic breakdown. Aldehydes are activated by proteins (a heat-stable system working particularly with acetaldehyde, and much faster with biliverdin than with methylene blue) and by an aldehyde dehydrogenase, probably different from xanthine oxidase. The other substrates mentioned above are activated by their specific dehydrogenases.

The bilirubin resulting from the reduction of biliverdin by liver enzymes was sometimes observed to possess the properties of "direct" bilirubin.

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CLXVI. PHOSPHATASE DISTRIBUTION IN SOME HIGHER PLANTS.

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THE important metabolic role of phosphorus is fairly well understood in the animal kingdom and in some of the fungi but is still relatively obscure in the higher plants.

Among the methods which might be used to obtain more information as to the significance of phosphorus for plant life is that of following the provision of mechanisms for its utilization by different parts of the plant at different stages of development. An attempt is made to adopt this method in the work reported. The most important mechanism for the utilization of phosphorus by plants and animals is believed to be the synthesis and hydrolysis of phosphoric esters by the group of enzymes termed phosphatases.

If it be granted that the availability of phosphorus for the plant does indeed depend on this mechanism then, since it is a general observation that the appearance of the necessary enzyme in biological situations is coincident as to time and place with the occurrence of its substrate and reaction products, a study of the presence and concentration of phosphatase in the different regions of the plant at successive stages of its life history would not only elucidate the role of phosphatase in the higher plants but might also provide data for an attack on the general problem of their phosphorus metabolism.

Unfortunately as yet we can seldom isolate enzymes in a pure form, nor can we determine them by purely chemical methods. The only practical way to study their presence quantitatively is to determine their activity, i.e. the rate at which they decompose suitable substrates, and this method has been used in this investigation.

Method.

Various extracting liquids were tested but in no case did any solvent remove the enzyme completely from the plant tissue. For this reason powdered tissue, in place of the usual extract, was incubated with the substrate. To obtain the powdered tissue fresh plant material was ground to a paste which was spread out in a thin layer on a clock glass and dried in a rapid current of air at room temperature. The dried material was then ground lightly to a fine powder. This procedure was found to be satisfactory and did not entail any serious loss of enzyme activity. The true dry-matter content of this powdered tissue was determined by heating aliquots in an oven at 56° to constant weight.

As substrate for the determination of the enzyme activity 5% sodium β -glycerophosphate was chosen for several reasons: (1) it is a naturally occurring compound, (2) it is stable in acid solution, (3) it is readily obtainable, (4) other investigators working with plant phosphatases have used it successfully.

The substrate solution was adjusted to pH 5.8 because, as seen from the figures in Table I, the optimum pH for the activity of bean phosphatase on sodium β -glycerophosphate lies between pH 5.5 and 5.9. Whilst the optimum pH

for the activities of the phosphatases of the other plants used in this investigation was not determined, the substrate was always adjusted to pH 5.8. This procedure will, however, not affect the validity of the conclusions because no attempt is made to compare phosphatase activities as between different plants.

Table I. *The effect of pH on the activity of plant phosphatase obtained by a chloroform-water extract of the "Canada Wonder" bean.*

Average pH	mg. inorganic phosphorus released. Time of hydrolysis	
	½ hour	2 hours*
4.2	0.12	0.33
4.6	0.20	0.63
5.0	0.29	0.84
5.5	0.32	0.96
5.9	0.33	0.94
6.2	0.30	0.87
7.2	0.10	0.26

* Average of two experiments.

For the quantitative estimation of phosphatase activity duplicate portions of 0.5 g. of the powdered tissue in 15 ml. of distilled water were brought to 37.5° in a water-bath. The substrate, 10 ml. of 5% β -glycerophosphate solution at pH 5.8, previously warmed to the same temperature, was then added. Three 3-ml. samples of each were taken at intervals of 5 min. and delivered instantly into 10 ml. of 7% trichloroacetic acid. The inorganic phosphate released by the action of tissue phosphatase was estimated by the Briggs [1924] colorimetric method in each duplicate. Control experiments were always conducted to determine the inorganic phosphate of the plant tissue itself. The amount of enzyme present is expressed as the number of mg. of inorganic phosphorus released in 1 min. by the action of the phosphatase present in 1 g. of dry plant tissue on 2% sodium β -glycerophosphate. This figure was obtained from the progress curve by interpolation.

Experiments were performed which show that the inorganic phosphorus released under these conditions, making due allowance for experimental error, is proportional to enzyme concentration, Table II.

Table II. *Relation of the plant phosphatase activity to its concentration.*

Amounts of powdered plant tissue used (g.)	Phosphorus released						
	Zero time	5 min.		10 min.		15 min.	
		mg.	x/c	mg.	x/c	mg.	x/c
0.07	0.00	0.87	12.4	1.50	21.5	2.19	31.2
0.06	0.00	0.74	12.3	1.37	22.8	2.00	33.3
0.05	0.00	0.67	13.5	1.22	24.4	1.75	35.0
0.04	0.00	0.46	11.6	0.95	23.7	1.42	35.5
0.03	0.00	0.38	12.8	0.75	24.9	1.08	36.0

x = Phosphorus released.

c = Weights of tissue.

EXPERIMENTAL PROCEDURE AND RESULTS.

Bean, potato, radish and wheat plants were used in these studies. Three different populations of beans were grown, two of the variety Canada Wonder and one of the Pencil Pod type. One population of Canada Wonder bean and the Pencil Pod beans were grown in soil. The second population of Canada

Wonder beans was grown in a culture solution. For the study of the initial stages of growth, however, the beans were germinated and grown in moist sawdust or cotton-wool.

The culture solution consisted of:

Calcium nitrate	4.0 g. anhydrous salt
Potassium nitrate	1.0 g. " "
Potassium dihydrogen phosphate	1.0 g. " "
Potassium chloride	0.5 g. " "
Magnesium sulphate	1.0 g. " "
Ferric chloride	0.0336 g. " "
Sodium citrate	0.648 g. crystalline salt
Distilled water	6 litres

Sodium citrate was used to form a soluble complex salt with iron and so to prevent the formation of the insoluble iron phosphate.

Canada Wonder beans grown in soil,

The results for the experiments on the Canada Wonder beans grown in cotton-wool and soil are shown in Table III.

Table III. *Phosphatase content of the different parts of Canada Wonder beans grown in cotton-wool.*

Days between planting and sampling	Part of plant	Phosphatase mg. P released by phosphatase in 1 g. of dry matter per min.
Ungerminated	Testa	—
	Cotyledon	Trace
	Germ	0.91
7	Testa	—
	Cotyledon	Trace
	Leaves	3.19
	Roots	4.89
12	Cotyledon	1.28
	Leaves	2.78
	Hypocotyl	5.05
	Roots	4.20
18	Cotyledon	2.36
	Leaves	4.65
	Stem	4.70
	Root	4.11
Canada Wonder beans grown in soil.		
21	Cotyledon	3.19
	Leaves	6.62
	Stem	4.51
	Root	2.84
28	Primary leaves	5.65
	Young leaves	4.65
	Stem I	3.45
	Stem II	3.13
	Roots	2.73

It will be noticed that no phosphatase was found in the testae. In the cotyledons little enzyme was detected during the initial stages of germination, but later an appreciable quantity of phosphatase appeared. As soon as the young plant had developed sufficiently to make separation into leaves and roots

possible, the root tissue was found to possess a greater phosphatase activity than the leaves. As the leaves developed, the phosphatase activity increased, whilst in the roots it declined. In the hypocotyl and the stem, as in the case of the roots, the concentration of enzyme was relatively high during the earlier stages of development of the plant and fell off rapidly as the plant matured. An experiment dealing with ungerminated seed was also carried out in the following way. The seed was soaked for 8 hours to soften the testae which were then removed. The embryo was dissected out from the cotyledons and the phosphatase activities of the testae, the cotyledons and the embryos were separately determined. It was found that the embryo alone showed any appreciable phosphatase activity, and only traces were found in the cotyledons. To judge the enzyme concentration in any particular tissue according to the phosphatase activity shown by that tissue may be open to the criticism that the possible presence of inhibitors is not taken into account. The following experiment was conducted to detect the presence of inhibitors in the cotyledons. Digest flasks were made up with both powdered cotyledon tissue and young bean plant tissue, and the phosphatase activity of the combined enzyme preparation was compared with the phosphatase activities of the cotyledons and the plant separately. The results are shown in Table IV.

Table IV. *The effect of cotyledon tissue on the activity of phosphatase present in other parts of the bean plant.*

Sources of phosphatase	mg. of phosphorus released			
	Zero time	5 min.	10 min.	15 min.
0.05 g. of powdered tissue of the young bean plant	0.00	0.86	1.47	2.17
0.05 g. of powdered cotyledons	0.00	0.26	0.53	0.79
0.05 g. of powdered tissue of the young bean plant, and 0.05 g. of powdered cotyledons	0.00	1.01	1.95	2.76

The figures show that the sum of the amounts of inorganic phosphorus released by the actions of the cotyledon and the plant separately was equal to the amount of phosphorus released by the combined actions of the cotyledon and plant. If inhibitory substances were present in the cotyledons, they would presumably have affected the activity of phosphatase derived from the young plant. As no such inactivation was demonstrated, it may be concluded that no inhibitors were present and that the apparent low activity of the enzyme in the cotyledons does indeed indicate that only small quantities of phosphatase are elaborated during the early stages of germination.

The possible effect of variation in the magnesium content in different parts of the plants on the enzyme activity must be considered. Munemura [1933] has shown that plant phosphatase, like animal phosphatase, is activated by magnesium. He showed that at the optimum *pH* the maximum increase in activity obtained is only of the order of 33 % above the dialysed control with 0.002 *M* MgCl_2 . Concentrations of MgCl_2 from 0.02 *M* to 0.00002 *M* in the digest did not vary the activity of the enzyme more than 25 %. As the variations in the phosphatase content found in the different parts of the plant were of the order of 100 %, the differences demonstrated could not be due to variation in magnesium content of the plant tissues. Latshaw & Miller [1924] and Fonder [1929] showed that variations in concentration of magnesium in different parts of the plant are comparatively small. In experiments preliminary to the work

here reported 0.002 *M* MgCl_2 in the digest of undialysed extract of ungerminated beans increased the activity of the enzyme only 10 %. The concentration of magnesium in digests may obviously vary greatly with no significant change in the activity of the phosphatase.

Canada Wonder beans grown in culture solution.

Phosphatase determinations on the population of Canada Wonder beans grown in culture solution were made at irregular intervals until the plants had completed their full growth and withered. The results of these determinations on the different parts of the plants are presented in Fig. 1.

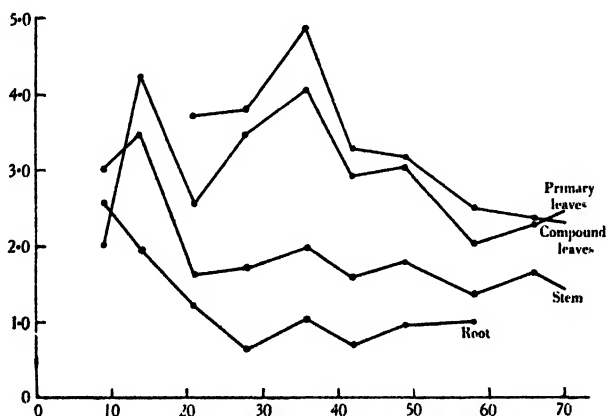


Fig. 1. Phosphatase activity of the bean plant grown in culture solution. Ordinate: mg. inorganic P released from sodium β -glycerophosphate solution by phosphatase in 1 g. of dry matter per minute. Abscissa: days after planting.

Except during the early period of growth there was little phosphatase activity in the roots. It amounted to only about 20% of that found in the leaves. The phosphatase concentration in the stem was low except in the early stages of growth, yet it was higher than in the root. The phosphatase activity in the stem, after falling to a low level at the end of the third week, remained at that level with slight variations throughout the growing period. The leaves appear to be the chief centres of phosphatase activity and, except for the first few days after germination, they have been shown to possess at least twice as much phosphatase activity as the stem tissue. The leaves were subdivided into two classes. The first class consisted of the primary simple leaves. In the second class were placed the compound leaves. At the first sampling, 9 days after the beans were planted, the phosphatase activity in the leaves was comparatively low, only two-thirds of that present in the stem. It must be borne in mind that at this early stage, the leaves are only partially developed and are still between the cotyledons. The true leaf function is not fully developed. 5 days later, when the primary leaves were well grown, the phosphatase activity had doubled, but as the plants became older it showed a gradual decrease.

Pencil Pod beans grown in soil.

Only a few pods were obtained from the beans grown in culture solution because flowers do not give rise to fruit very readily under these conditions. For this reason a population of beans was grown in soil during its entire life

history. The variety "Pencil Pod" was used in this set of experiments because this variety grows more vigorously and more evenly than the Canada Wonder beans under greenhouse conditions. The results of the determinations made on Pencil Pod beans are given in Table V.

Table V. *Phosphatase content of the different parts of Pencil Pod beans grown in soil.*

Days between planting and sampling	Part of plant	Phosphatase mg. P released by phosphatase in 1 g. of dry matter per min.
13	Leaves	5.18
	Stem	1.93
	Root	1.29
20	Primary leaves	2.48
	Compound leaves	2.72
	Stem	0.67
	Root	0.74
40	Flowers	1.02
	Primary leaves	2.85
	Compound leaves	2.61
	Stem	0.63
52	Flowers	1.76
	Younger pods	1.51
	More mature pods	1.47
	Leaves	1.66
	Stem	0.68
59	Beans	0.47
	Pods	1.08
	Leaves	1.54
	Stem	0.66
72	Cotyledons without plumules	0.32
	Cotyledons with plumules	0.33
	Leaves	1.08
	Stem	0.87
	Root	0.52

The phosphatase distribution was found to be very similar to that found in the population grown in the solution. In these experiments it was possible to make two determinations on flowers and pods. The results show that phosphatase is present in these parts of the plant. It is present, in fact, in quantities which are much greater than those found in the stem or root during the later stages of its life history, although only one-fourth to one-fifth of the maximum amounts found in the leaf. The developing bean itself contains very small quantities of the enzyme. One experiment was conducted in which the newly forming embryo was removed from between the newly forming cotyledons, and the concentrations of phosphatase in the separated cotyledons were compared with the phosphatase content of the intact bean. No significant differences were obtained in the phosphatase activities of the two preparations.

The potato tuber.

In the bean experiments the greatest phosphatase concentration was found in the leaves. This fact suggested that the enzyme might be connected with the carbohydrate metabolism of the plant. It was of importance to determine whether the concentration of phosphatase varies with the change of reserve carbohydrate, such as starch, to a more soluble and utilizable form; especially since phosphorus is always associated with this polysaccharide. Phosphatase

activity in potato tubers was therefore determined. Analyses were made twice, 36 and 44 days after planting when shoots were well developed. The tubers were planted in sawdust instead of soil to facilitate the removal of the entire plant at sampling, and were kept in the greenhouse. In determining the phosphatase activity of the tuber the outside corky tissue was removed because it would have been erroneous to consider this inert material as part of the metabolically active tissue. The results of the experiments are reported in Table VI.

Table VI. *The phosphatase contents of the different parts of potato grown in sawdust.*

Days between planting and sampling	Part of plant	Phosphatase mg. P released by phosphatase in 1 g. of dry matter per min.
36	Tuber	Trace
	Leaves	1.45
	Stem	0.81
	Root	Trace
44	Tuber	0.28
	Leaves	1.23
	Stem	0.46
	Root	0.17

The distribution of phosphatase was found to be similar to that found in the bean. The phosphatase concentration is greatest in the leaves, the activity of the enzyme in the stem is weaker, whereas in the tuber and the roots the activity of phosphatase falls to a very low level. It appears therefore that phosphatase is not concerned with the process of transformation of starch into a less complex carbohydrate since the enzyme was present in very small concentrations in the tubers where this process takes place.

The radish.

It would have been of interest to study the phosphatase content of the potato tuber during the reverse process, that is during the period of deposition of starch, but practical difficulties precluded the performance of this experiment. Instead, experiments were conducted with radishes to determine whether, during the deposition of carbohydrate food reserve in the swollen part of the hypocotyl, the phosphatase increases in that region. A variety of radish was selected that would produce a "bulb" under greenhouse conditions. The seed was planted in light soil in wooden boxes, and after 10 days the small plants were planted out to allow unrestricted development. Samples were taken at this stage and the results are given in Table VII.

The leaves had the highest concentration of phosphatase. The petioles had half that concentration, and in the root it was about 40% lower than in the petioles. 2 weeks later another sample was taken. The plant was subdivided into the leaf laminae, the petioles, the swelling hypocotyl and the roots. The leaf again was shown to have the highest phosphatase activity, whereas in the petioles and the swelling hypocotyl it was much weaker. The enzyme activity in the root was weaker than in the petiole. When the "bulbs" were well developed 2 weeks later another sample was taken. Allowing for experimental error, no change in the concentration of the enzyme in the different parts was noticed. If the phosphatase concentration in any organ is taken as a criterion of its importance for the metabolic process of that organ, the results of the experiments on radishes indicate that phosphatase plays no important part in the deposition

Table VII. *The phosphatase content of the different parts of radishes grown in soil.*

Days between planting and sampling	Part of plant	Phosphatase mg. P released by phosphatase in 1 g. of dry matter per min.
11	Leaves	3.84
	Petioles	1.68
	Root	1.15
25	Leaves	2.20
	Petioles	1.37
	Swollen hypocotyl and root	1.03
	Root	0.94
38	Leaves	2.13
	Petioles and stem	1.73
	Swollen hypocotyl and root	0.99
	Root	1.09

of sugar in the swollen hypocotyl, for the concentration of the enzyme in this part of the plant was never higher than in the petiole which is morphologically similar to it.

Wheat.

The plants so far studied, bean, radish and potato, belong to the dicotyledons. It is of interest to learn whether the distribution of phosphatase in the monocotyledons is similar.

Wheat of the variety Marquis was selected as representative of the monocotyledons. It was impossible to continue the experiments with wheat to the stage of maturation because the entire wheat population was attacked by loose smut soon after "heading". Three plants were grown in each pot. 2 weeks after seeding, samples were taken and phosphatase activity was determined on three portions of the plant: (1) the leaf laminae, (2) the leaf sheath and all the tissues enveloped by the leaf sheath, (3) the root. In the root it was found to be very low (Table VIII).

Table VIII. *The phosphatase content of the different parts of Marquis wheat grown in soil.*

Days between planting and sampling	Part of plant	Phosphatase mg. P released by phosphatase in 1 g. of dry matter per min.
14	Laminae of leaves	0.79
	Sheath	0.98
	Root	0.56
45	Laminae of leaves	0.76
	Sheath	0.82
	Root	0.24
61	Head	0.89
	Leaves	0.46
	Sheath	0.47
	Stem	0.50

The concentration of phosphatase in the leaf sheath was higher and equal to that in the laminae. 31 days later the same subdivision of the plant was made. The results did not differ greatly from the previous sampling.

16 days later, when the wheat was headed, another sample was taken. The concentrations of phosphatase in the head, the leaf laminae, the leaf sheath and the stem were determined. The leaf sheaths, the leaf laminae and the stem all

gave approximately equivalent results; the enzyme concentration in the head was however considerably greater than in the other parts. In sampling, the whole head was ground up, as it was impossible to obtain enough material to subdivide the head into its component parts. The relatively high concentration of phosphatase in the head is of interest, but unfortunately only one determination was possible owing to the destruction of the crop.

Wheat was planted in sawdust to study the early stages of development. The relative distribution of phosphatase in the different parts of the plant was similar to that found in the wheat grown in the soil, although the amounts were all larger, Table IX, because the samplings were performed during the earlier

Table IX. *The phosphatase content of the different parts of Marquis wheat grown in sawdust.*

Days between planting and sampling	Part of plant	Phosphatase mg. P released by phosphatase in 1 g. of dry matter per min.
4	Grain	0.52
	Aerial portion	1.40
	Root	0.99
6	Grain	1.00
	Aerial portion	1.79
	Root	1.11

stages of development. Enzyme concentration was determined on the different parts of the sprouted grain 4 days after planting. At this stage the roots were well developed and the first leaf was just protruding beyond the leaf sheath. The figures show that the sheath with the developing leaf had the highest phosphatase concentration. The enzyme was less concentrated in the roots, and the grain had about half the amount of phosphatase found in the root. 2 days later another sample was taken. Increases in enzyme concentration in the plumule and the remains of the grain were noticed. The wheat plant as a whole appeared to have a relatively high concentration of phosphatase during the first few days of germination, the amount of enzyme then decreasing; the decrease being first noticed in the roots. These observations are in accordance with results published by Hirsch [1931] and by Ay [1930].

DISCUSSION.

The methods used in obtaining the results reported were always similar and therefore the concentration of phosphatase which was found in any part of a plant can be compared with those found at different stages of the life history of the same or other parts of the plant. Although plant tissues have highly generalized functions and the metabolic processes are common to most cells, yet the intensity of these processes varies in the different parts of the plant during the various stages of its life history. Furthermore photosynthesis takes place only in the green portions of the plant.

Several workers have reported the importance of phosphates in plant carbohydrate metabolism. Lyon [1924] and Chatterjee [1933] have found that the rate of respiration in plants is increased by phosphate. Cockfair [1931] and Barrenscheen & Pany [1930] have shown the presence of hexosephosphates in assimilating plants. Cockfair showed in the tomato plant that, during assimilation in the light, hexosephosphates increased in concentration and during darkness they decreased.

It would thus appear that phosphates play an important role in both the catabolism and anabolism of the carbohydrates in the higher plants. The photosynthesis or the anabolic process of carbohydrate metabolism in the plants takes place only in the green portions of the plant and chiefly in the leaves. The leaves, especially during the earlier part of their development, are the parts of the plant which have the greatest rate of respiration, and it was in the leaves of the beans, the potato and the radish that the phosphatase activity was found to be greatest.

As the highest phosphatase concentration in the plant is found in the leaves, the site of vigorous carbohydrate metabolism, it is reasonable to assume that phosphatase as well as phosphates may play a role in the carbohydrate metabolism of the higher plants.

In the wheat plant it was found that the phosphatase concentrations in the leaf laminae, leaf sheaths and stem are approximately equal to one another. Morphologically and physiologically the leaf sheath and the leaf are very similar to each other. The stem, on the other hand, does not play an important role in photosynthesis, but in the case of wheat it contains a considerable amount of meristematic, quickly growing and rapidly respiring tissue at the nodes. Actually the phosphatase concentration of the stem was equal to that of the leaf sheaths and leaf laminae but, since the experiments with the wheat population were not conducted to the stage of maturation of the crop, it is possible that the phosphatase concentration in the stem would have dropped appreciably below that of the leaves after the stem had completed its growth.

The phosphatase concentrations in the stem and petioles of the bean, potato and radish were lower than that in the leaves. It was found that, in the case of the bean plant which was studied more thoroughly than the other plants, the phosphatase concentration of the hypocotyl was comparatively very high. As the stem matured, however, the phosphatase concentration decreased greatly although it was always higher than the concentration of the enzyme found in the roots. The high concentration of phosphatase in the hypocotyl may probably be explained by the fact that at that stage of development this portion of the plant is growing very rapidly and consequently respiring very actively.

These results conform generally with the hypothesis that phosphatase may be concerned with carbohydrate metabolism. The concentration of phosphatase in the roots of the bean, potato, radish and wheat is lower than that found in the leaves and stem. The actual presence of phosphatase in the roots can be explained if one accepts its role in respiration, but the enzyme may have a further function. Heck & Whiting [1927] and Weissflog & Mengdehl [1933] have shown that organic phosphorus compounds are readily available to plants, and it is possible that the root phosphatase may be concerned in the process of absorption by the plant of these organic compounds from the external medium.

Although the experiments reported in this paper were concerned with the distribution of phosphatase in the plants, the mere study of relative concentrations of the enzyme in the various parts of the plant at different stages of the life history was not the objective. As has been already pointed out in the introduction, the work was undertaken to throw more light on the role of phosphatase and consequently on the phosphorus metabolism of the plant. On the basis of the experimental results it seems possible that phosphatase plays a part in the carbohydrate metabolism of the higher plants, but further work is necessary to confirm the validity of the suggestion. The significance of the work reported lies in the fact that it is possible to show that phosphatase is active in the plant throughout its life history. Although the presence of phosphatase in the leaves had been previously known, most of the work which could throw some

light on the physiological role of the enzyme was carried out on ripening and germinating seeds. From the work of other investigators an impression had been created that phosphatase is concerned with the synthesis of organic phosphorus compounds as a store of that element in the ripe seeds, and that during germination the enzyme hydrolyses these organic compounds to release the phosphate radical for the use of the developing embryo. Phosphatase is, however, present in all parts of the plant and the fully developed leaves have the greatest concentrations. It must therefore play an important part in the metabolism of the plant throughout the whole of its life cycle.

SUMMARY.

Analyses of beans, potatoes, radishes and wheat for phosphatase content were made and the results are reported.

The bean. 1. Phosphatase activity in the dormant bean seed is low. The embryo contains a much higher concentration of the enzyme than the cotyledons.

2. In the germinated seed the enzyme concentration is much greater than in the dormant seed. The hypocotyl and the stem have the greatest concentration of enzyme, but the concentration falls off rapidly after a short period of growth. The concentration in the roots is not quite so high as in the stem. In the leaves the phosphatase concentration is low, but it is higher than that found in the cotyledons. The very low concentration of phosphatase in the cotyledons during the early stages of germination is not due to the presence of inactivators.

3. In the leaves, after they have emerged from between the cotyledons, the phosphatase activity gradually rises and reaches a maximum after a few days. The concentration of enzyme in the leaves at its optimum is higher than that of any other part of the plant throughout its entire life history.

4. As the plant matures the phosphatase concentration in the leaves decreases gradually, but in the stem and root it falls very rapidly, although it is always higher in the former than in the latter.

5. In the flowers the enzyme concentration is low and is comparable with that found in the leaves of a fully matured plant. The phosphatase content of the pods is equal to that of the flowers. The newly forming beans have a very low enzyme concentration.

The potato. In the potato plant which is sprouting from the tuber the greatest enzyme concentration is found in the leaves, and much smaller concentrations are found in the stem, whereas in the tuber and roots only slight traces can be detected by the experimental methods employed.

The radish. In the radish phosphatase is present in all parts of the plant, the greatest concentration being found in the leaves. In the petioles there is also a considerable amount of the enzyme. The root and the swollen hypocotyl have much smaller concentrations of phosphatase than the petioles. The radishes were grown for one month, during which time three samplings were made. The results did not vary throughout this period.

Wheat. 1. In the wheat grain the enzyme increases in concentration during germination. The greatest phosphatase content, however, is found in the plumules. The concentration of the enzyme in the rootlets was intermediate between that of the grain and the plumule.

2. When the aerial portion could be subdivided into two parts, the laminae and the leaf sheath with the tissues they enclosed were examined. No significant differences were noticed in the enzyme concentrations of those two parts of the plant.

3. At heading the phosphatase concentrations in laminae, leaf sheath and stem are equal and are lower than that found in the head.

4. The trend of phosphatase concentration in the life history of the wheat plant is similar to the trend in the bean plant, for the concentration of the enzyme in the young plants is high and decreases to a low level during maturation.

The writers are indebted to Prof. G. H. Duff of the Department of Botany for his interest and advice during the progress of the experiments.

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CLXVII. ON THE HYDROLYSIS OF GUANINE.

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(Received 23 May 1936.)

SOME two years ago when examining certain autoclaved material by the diazo-test for thymine recently described [Hunter, 1936], a greenish blue colour was observed as soon as the test solution was mixed with the alkaline diazo-reagent. The colour faded rapidly to yellow in the presence of the weak alkali, but if a few drops of sodium hydroxide were added quickly after the test solution, the green colour changed to a beautiful blue which was stable for many hours. In the writer's experience such a test was unique and seemed to call for further investigation. A small amount of commercial yeast nucleic acid was heated with 25 % sulphuric acid at 150–160° for 5 hours (the usual conditions for the hydrolysis of nucleic acid to yield pyrimidines) and a little of the neutralized hydrolysate tested with the diazo-reagents; a strong positive test was obtained. Adenine and guanine were then separately hydrolysed in a similar manner with the result that the guanine hydrolysate yielded a very strong test, whilst the adenine hydrolysate was quite negative. It was thus established that guanine was the source of the substance giving the blue colour with the diazo-reagents.

Experiments were made with quantities of 10–50 mg. guanine hydrochloride to establish the optimum conditions of strength of acid, time of heating and temperature, for the production of maximum yields of the substance as measured by the colour given by the diazo-reagents. Great accuracy could not easily be attained and was not aimed at, the best conditions being finally somewhat arbitrarily decided upon. In the earlier work sulphuric acid was employed, but to simplify the isolation hydrochloric acid was later substituted.

The unknown substance was apparently very stable in acid solution; it was not precipitated by silver in the purine fraction, but was precipitated quantitatively with silver and barium hydroxide and was recoverable by the usual H_2S treatment; it was also precipitable by mercuric chloride near neutrality and by phosphotungstic acid; although its effective recovery from the phosphotungstate was rendered doubtful by the blackish blue colour given in excess of $\text{Ba}(\text{OH})_2$. Means, in short, seemed to be available for the separation of the substance from both purines and pyrimidines, but since pure guanine was employed pyrimidines were not likely to complicate the isolation. As the sequel will show, no metallic precipitant is actually necessary in the isolation of the pure substance.

The first crystalline material was obtained as sulphate from the silver-baryta precipitate. When excess of absolute alcohol was added to the concentrated silver-free fraction acidified with H_2SO_4 , an oil separated. After cooling and scratching with a glass rod discrete well-formed crystals appeared. The material thus obtained, however, was not ash-free, and after repeated crystallization it still retained traces of what appeared to be silica. The experience of Fischer [1910], who obtained a 50–60 % yield of xanthine by boiling guanine for 30 hours with 20 % HCl was similar to this, in that his xanthine was always contaminated with silica, which was avoided only by recourse to a platinum vessel for hydrolysis.

The main bulk of the base has, however, been prepared as hydrochloride. The dihydrochloride crystallizes readily by the method described below, and there is no difficulty in obtaining ash-free preparations. From various batches totalling 40 g. guanine hydrochloride, 7.2 g. of the dihydrochloride of the new base $C_4H_7N_5 \cdot 2HCl$ were obtained, a yield of 15.6%. A special batch of 6.21 g. guanine hydrochloride, the quantitative degradation of which is reported below yielded 1.16 g. $C_4H_7N_5 \cdot 2HCl$, or just over 16% on a weight basis.

*Analyses.*¹

Sulphate. Found: C, 21.93, 21.60; H, 4.35, 4.26; N, 30.75, 31.19, 30.87; S, 14.28, 14.29; ash, 0.36%. $C_4H_7N_5 \cdot H_2SO_4$ requires C, 21.52; H, 4.03; N, 31.40; S, 14.35%.

Dihydrochloride. Found: C, 24.41, 24.34; H, 4.48, 4.58; N, 35.10, 35.33, 35.17; Cl, 36.06, 36.05%. $C_4H_7N_5 \cdot 2HCl$ requires C, 24.24; H, 4.58; N, 35.38; Cl, 35.81%.

Evidence pointing to the constitution of the new base as 4- (or 5-) guanidinoglyoxaline is presented in the following paper.

A small amount of the new base remaining in the mother-liquors from the above preparation was precipitated, after removal of chloride, by silver and baryta at pH 8.0. Large excess of baryta was then added to the filtrate and a further appreciable precipitate was obtained, which was decomposed, the solution being freed from Ag, Ba and SO_4 , evaporated *in vacuo* to small volume and made acid with HCl. By treatment with alcohol about 60 mg. of small well-formed rhombohedra, m.p. 200°, were obtained, which proved to be glycoeyamine hydrochloride. Addition of picric acid to the filtrate from this yielded a mixture of the picrates of guanidine and glycoeyamine.

The filtrate from the silver-baryta precipitate was treated with H_2S in the usual manner; excess of HCl was added and the material taken to dryness to get rid of HNO_3 . By treatment with alcohol 0.63 g. of white spicular crystals was obtained melting fairly definitely at 180°. The filtrate brought to dryness *in vacuo* and taken up in hot absolute alcohol gave another crop of crystals, m.p. 147°.

On micro-analysis (Dr Stantial) the two substances gave respectively: C, 21.80; H, 5.51; N, 12.99; Cl, 31.73% and C, 17.54; H, 4.34; N, 20.86%. They were thus glycine hydrochloride (requires C, 21.52; H, 5.38; N, 12.57; Cl, 31.80%) and glycine nitrate (requires C, 17.39; H, 4.35; N, 20.29%).

A small amount of substance having 11.42% N and m.p. 225° had been isolated from the same fraction, which proved to be glycine sulphate (m.p. 225°, 11.29% N). The latter had arisen from a slight excess of H_2SO_4 left when removing Ba.

The disintegration of guanine having thus been fairly exhaustively studied (all products with the exception of glycoeyamine having at this stage been identified) it was thought worth while to fractionate a hydrolysate as quantitatively as the removal of test portions at various stages permitted. The following summarized account is described in detail in the experimental section.

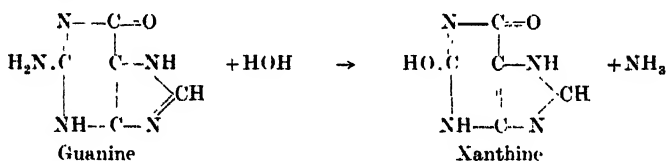
It is clear from this account that any degradation product of guanine that may have escaped detection must be present in very small amount. Considering the relative complexity of the analysis and the frequent removal of small test portions of which no account was kept the recovery is for practical purposes complete.

¹ Micro-analyses performed through the courtesy of Prof. W. Lash Miller, Department of Chemistry, University of Toronto, by Dr Helen Stantial.

Material used:	g. N	% Total N
6.21 g. guanine hydrochloride = 4.574 g. guanine	2.120	100
<i>Recovery, in order of separation:</i>		
1. 2.402 g. Xanthine (gravimetrically)	0.885	41.8
2. Ammonia-N (by titration)	0.600	28.3
3. 1.26 g. C ₄ H ₇ N ₅ .2HCl (colorimetric estimation)	0.446	21.0
1.16 g. C ₄ H ₇ N ₅ .2HCl isolated as such	—	—
0.07 g. C ₄ H ₇ N ₅ .2HCl isolated as dipicrate	—	—
i.e. 1.23 g. C ₄ H ₇ N ₅ .2HCl isolated = 97.6% of estimated	—	—
4. 0.326 g. Glycine (by ninhydrin and amino-N determination)	0.061	2.8
0.180 g. Glycine isolated as glycine sulphate = 55% of estimated	—	—
5. 0.037 g. } Guanidine. Colorimetric estimation	0.026	1.2
6. 0.037 g. } Glycoeyamine.	—	—
0.026 g. isolated as mixed picrate = 70% of estimated	—	—
Total accounted for	2.018	95.1

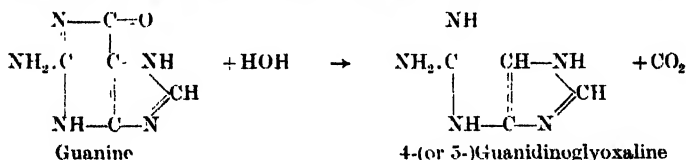
DISCUSSION.

When guanine is heated with strong acids under the conditions described the changes taking place are more complex than have been previously recognized. It is clear that the main reaction is:



As shown 4.574 g. guanine yielded 2.402 g. xanthine or a yield of 52%. This is comparable with the yields obtained by Fischer¹ [1910].

Proceeding alongside this reaction is the apparent removal of the CO group in position 6 with the production of the new base assumed (see following paper) to be 4- (or 5-) guanidinoglyoxaline and CO₂ according to:



As shown 4.574 g. guanine yielded 1.26 g. guanidinoglyoxaline dihydrochloride = 0.796 g. of the free base. For the production of 0.796 g. guanidinoglyoxaline 0.962 g. guanine is required; so that the reaction outlined above proceeds to the extent of 21.5% on the basis of guanine used.

If the guanine utilized for the formation of xanthine and of guanidinoglyoxaline (2.386 + 0.962 = 3.348 g.) be deducted from the total guanine used, (4.574 g.) there is left 1.226 g. guanine, available for the production of glycine, guanidine, glycoeyamine and ammonia. From 1.226 g. guanine there could theoretically be produced 0.609 g. glycine. The yield of glycine actually obtained, namely 0.326 g., thus represents 53% of that obtainable from the guanine available for its formation, or just over 14% yield from the total guanine employed. The production of glycine is thus not insignificant from a quantitative

¹ It should be noted that by Fischer's method of hydrolysing for 30 hours with 20% HCl at 100°, an appreciable amount of the base giving the blue diazo-test is formed.

aspect. It appears probable that 4-(or 5)-guanidinoglyoxaline is an intermediate product in its formation (*vide* p. 1190).

The guanidine theoretically derivable from 1.226 g. guanine is 0.479 g. compared with a yield of about 0.030 g., about 6% on the basis of guanine available or less than 2% of the total guanine. The glycoeyamine obtained is even less and is unlikely to represent more than 1% of the available guanine. It is not unlikely that both products are derived through 4-(or 5)-guanidinoglyoxaline.

The total ammonia-N produced is recorded above as 0.60 g. Of this 0.22 g. is produced when 2.402 g. xanthine are formed. There thus remains to be accounted for 0.38 g. N. There is this amount of N in 0.82 g. guanine. If 0.82 g. be subtracted from 1.226 g. there is left 0.406 g. guanine. From this there is theoretically obtainable only 0.203 g. glycine which is less than that (0.326 g.) actually obtained; from which it can be concluded that one N of the glyoxaline ring takes part in the formation of glycine while the other forms ammonia.

EXPERIMENTAL.

In all experiments a well crystallized commercial product sold as guanine hydrochloride was used. This showed a loss of 8.93% moisture on heating at 105°, and by micro-Kjeldahl 34.70% N (calculated for $C_5H_7N_5O \cdot HCl$, H_2O , 8.76% moisture and 34.10% N).

6.21 g. guanine hydrochloride were put in a short-necked 300-ml. Kjeldahl flask with 250 ml. 3.4 N HCl. A small beaker was inverted over the opening and the flask placed in a gas-heated autoclave; 23 min. were required to raise the temperature to 158°, at which point it was held for 90 min.; the flame was then extinguished and after 65 min. the temperature had fallen just below 100° when the autoclave was opened and the flask was removed.

The contents were transferred with washings to a 500 ml. balloon flask and distilled *in vacuo* to dryness. About 50 ml. water were added and again distilled off. This was repeated. Most of the HCl was thus removed. Finally about 75 ml. water were added, and the contents were left in a refrigerator overnight.

The precipitate, which is practically pure xanthine, was carefully collected on a small Hirsch funnel, well washed with water and dried; wt. 2.402 g. The material gave a picrate and a nitrate identical with the corresponding salts prepared from authentic xanthine. Its behaviour with the murexide test was also typical of that of xanthine.

The filtrate and washings from the xanthine were brought to 100 ml. 0.5 ml. was removed and diluted to 5 ml. for ammonia-N determination by aeration, and by micro-Kjeldahl distillation. The aeration method yielded 0.586 mg. N/ml.; the distillation method 0.61 mg. N/ml., or an average of 0.6 mg. N/ml. of the 1 in 10 dilution.

1 ml. of the same solution contained 2.94 mg. Cl; the whole solution thus containing nearly 3.0 g. Cl required rather more than 7.0 g. $Ba(OH)_2$ for neutralization.

The solution was again transferred with washings to a 500 ml. balloon flask and 7.8 g. finely powdered $Ba(OH)_2$ were added. Distillation *in vacuo* was continued until a drop of the contents of the flask no longer gave a Nessler reaction. When the solution had thus been freed from NH_3 and the volume brought to about 100 ml. by addition of water, the calculated amount of 5N H_2SO_4 was added to precipitate the Ba. The solution was freed from both Ba and SO_4 ions by final adjustment with dilute H_2SO_4 and $Ba(OH)_2$. The mixture was centrifuged and the $BaSO_4$ well washed with water. The fluid and washings were transferred to a 500 ml. balloon flask and evaporated *in vacuo* to about 15 ml. The slightly brown solution was brought to the boil, treated with a very small amount

of powdered charcoal and filtered through a small funnel into a boiling-tube, the paper being well washed with hot water. It was then evaporated *in vacuo* to about 7 ml. when solid began to appear on the sides of the tube. Evaporation was continued until crystals appeared in the body of the fluid. The tube was then removed from the pump, heated over a flame to dissolve the crystals, and absolute ethyl alcohol was added from a pipette until crystals appeared in the still warm solution (about 10 ml. alcohol). The tube was then cooled under the tap, 5–7 ml. alcohol were added, and the tube was left in the refrigerator overnight. The crystals were collected, washed with absolute alcohol and dried at 105°; wt. 1.003 g. The filtrate was concentrated, again treated with charcoal and yielded a second crop of white crystals, 0.114 g. The material even of the second crop was practically pure 4-(or 5)-guanidinoglyoxaline dihydrochloride, and represents at this stage more than 90 % of that estimated by colour test.

From the diluted aliquot used above for NH_3 and Cl determinations, a portion was removed and again diluted 10 times. A standard solution containing 0.1 mg./ml. pure 4-(or 5)-guanidinoglyoxaline dihydrochloride was prepared. Into each of two tubes were put 5 ml. 1.1 % sodium carbonate and 2 ml. Koessler and Hanke diazo-reagent. To one tube was added 0.5 ml. of the standard, to the other 0.5 ml. of the unknown, followed after mixing in each case by 1 ml. 2.5 N NaOH. Compared in a Duboscq colorimeter the test read 15.0 mm. against the standard at 18.9 mm., so that the amount of dihydrochloride present was 1.26 g.

The filtrate and washings from the second crop of the dihydrochloride were distilled *in vacuo* to remove alcohol, and made to a vol. of about 30 ml. with water. A solution of 10 % AgNO_3 was then added until a drop of the mixture showed slight blackening with a solution of $\text{Ba}(\text{OH})_2$ on a tile. The precipitate was centrifuged, washed with a little water and discarded. To the fluid a hot solution of $\text{Ba}(\text{OH})_2$ was very carefully added to pH 8.0 (bromothymol blue). The precipitate was separated, washed with a little water, transferred to a small flask, made acid with H_2SO_4 , treated with H_2S and stoppered (*Fraction A*). The filtrate was made strongly alkaline with a hot saturated solution of $\text{Ba}(\text{OH})_2$, the precipitate was separated, washed with a solution of $\text{Ba}(\text{OH})_2$, transferred to a small flask, made acid with H_2SO_4 , treated with H_2S and stoppered (*Fraction B*). The silver-baryta filtrate was transferred to a flask, made acid with H_2SO_4 and treated as above (*Fraction C*).

Fraction A was freed from Ag_2S , and from H_2S by distillation of the acid solution *in vacuo*. Ba and H_2SO_4 were removed as usual. To a volume of about 10 ml. about 5 ml. of hot saturated aqueous picric acid were added. The copious picrate which separated was dissolved by heat and the tube allowed to cool. It was separated and dried; wt. 0.205 g., M.P. 200°. After recrystallization from water the M.P. was 216°, or the same as that of the dipicrate prepared from pure 4-(or 5)-guanidinoglyoxaline (*vide* p. 1193).

Fraction B was treated as fraction A, the Ba- and H_2SO_4 -free solution being strongly alkaline to litmus. To a volume of about 25 ml. were added 15 ml. of saturated aqueous picric acid. The precipitate was dissolved by heat and left overnight to crystallize. Under the microscope there appeared two crystalline species distinguished by both form and colour. The mixed picrate was filtered and weighed 170 mg., M.P. 220–225°. The filtrate was concentrated and a crop of about 8 mg. sulphur-yellow needles, M.P. 200°, was obtained. The first crop was recrystallized from water and quickly yielded crystals typical of guanidine picrate, M.P. 325°. The crystals were of a deep golden colour with zigzag or swastika forms. The sulphur-yellow needles on recrystallization had M.P. 207°, the melt being typically blood-red in colour.

It has been mentioned that in the treatment of this fraction obtained from 40 g. guanine hydrochloride, a small amount of the hydrochloride of a base, M.P. 200°, had been obtained. This substance was now found to form a picrate similar in all respects to that here described. Despite some difference in M.P. of the hydrochloride and the picrate recorded for glycoeyamine, this substance was suspected. It was accordingly synthesized by the method of Ramsay [1908] from guanidine and chloroacetic acid. Its hydrochloride was found to melt at 200°, its picrate at 207° and its picolonate at 245°. In crystalline form and colour all the derivatives of our base were identical with the corresponding ones from glycoeyamine. By heating a few mg. of the hydrochloride of glycoeyamine, and a few mg. of the hydrochloride in question to 170° and dissolving in a little water, a strong Jaffé test was obtained from each solution. The Weyl test, characterized by a burgundy colour on acidification [Barger, 1914], was equally good evidence for the conversion into glycoeyamidine.

An attempt was made to estimate the guanidine in fraction B, before the treatment with picric acid just described. The volume of the solution had been brought to 25 ml. A standard solution of guanidine carbonate containing 1 mg./ml. was made. The Marston modification of the Tiegs test was used.¹ It was found that 0.1 ml. of the test solution gave approximately the same colour as 0.3 ml. of the standard guanidine carbonate. The 25 ml. of fraction B thus contained the equivalent of 75 mg. guanidine carbonate or 37 mg. guanidine. The estimate is a maximum as the mol. wt. of glycoeyamine is double that of guanidine.

Fraction C was treated as fractions A and B, taken to a small volume and made to 25 ml. Preliminary tests with ninhydrin indicated that the solution contained about 12 mg. glycine per ml. Two determinations in the Van Slyke micro-amino-N apparatus showed the presence of 2.40 mg. $\text{NH}_2\text{-N/ml.}$ or 0.060 g. in fraction C. This amount corresponds to 0.326 g. glycine.

Very slightly more than the theoretical amount of H_2SO_4 was now added to about 23 ml. remaining from the solution mentioned. It was evaporated *in vacuo* in a small tube to a vol. of 1–2 ml. and absolute alcohol was added. The crystals were filtered off; yield 0.183 g., M.P. 226°, identical with the sulphate made from glycine. A further crop of 0.107 g. was obtained giving a total of 290 mg. glycine sulphate against a theoretical yield of 496 mg. glycine sulphate.

I am indebted to Mr Elly Margolis, who assisted with the preliminary experiments during the summer of 1934, and to Mr Isydore Hlynka, who has largely carried out the preparations of the guanidinoglyoxaline for the past year. Grateful acknowledgement is made to the Carnegie Research Grant Committee of the University for financial aid.

¹ The reagent used here was Weber's modification of Marston's reagent as described by Robertson [1929], and is made by mixing in order 5 ml. 10% sodium nitroprusside, 5 ml. 10% potassium ferricyanide, 5 ml. 10% sodium hydroxide, with the final addition of 45 ml. water. The test is carried out by adding to about 1 ml. unknown solution, made alkaline with 1 drop 2.5 N NaOH, 0.5 ml. of the reagent after it has been kept for 30 min.

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CLXVIII. A NEW GUANINE HYDROLYTIC PRODUCT [4-(OR 5-)GUANIDINOGLYOXALINE], AND ITS HYDROLYTIC PRODUCT [4-(OR 5-)CARBAMIDOGLYOXALINE].

By GEORGE HUNTER.

WITH A NOTE ON THE DISSOCIATION, AND ULTRAVIOLET ABSORPTION SPECTRA.

By WILLIAM DOUGLAS McFARLANE.

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(Received 23 May 1936.)

THE formulation of the new base obtained from guanine by hydrolysis as 4-(or 5-)guanidinoglyoxaline [Hunter, 1936, 1, 2] is based on the evidence which will now be submitted.

Prima facie its formation as a simple hydrolytic product of guanine through the removal as CO_2 of C in position 6, with simultaneous reduction of the adjacent N and C atoms, seems probable.

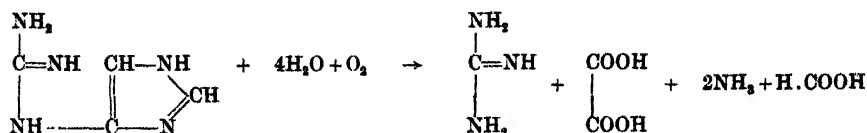
That the base retains the glyoxaline ring of the original guanine is indicated by its readiness to couple in sodium carbonate solution with sodium diazo-benzene-*p*-sulphonate. The formation of a blue coupled product is unique among the known glyoxalines but a side-chain of the nature of guanidine might well lead to such a colour.

With the Sakaguchi test the base yields an intense green-blue colour, in contrast to the red typical of monosubstituted guanidines. After oxidation with bromine or potassium permanganate in an acid medium, the solution fails to give a Sakaguchi test but responds positively to Tiegs's test; which is evidence for the appearance of guanidine and the absence of a substituted guanidine. After oxidation with permanganate, about 75 % of the guanidine theoretically derivable from the base has been isolated as picrate, and after oxidation with bromine the guanidine released according to colorimetric estimation with Tiegs's reagent is over 90 % of the theoretical amount.

After oxidation, the diazo-test on the solution is negative, which suggests disruption of the glyoxaline nucleus. Along with guanidine there may readily be detected in solution oxalic acid,¹ ammonia, formic acid and a small proportion of

¹ The appearance of oxalic acid in a solution resulting from permanganate oxidation needs comment. The explanation appears to be that as the oxalic acid is produced the formation of an exceedingly insoluble guanidine oxalate removes it from the sphere of the reaction. This compound was first observed following the addition of slight excess of bromine to a solution of $\text{C}_4\text{H}_7\text{N}_5 \cdot 2\text{HCl}$, but it may also be observed in either a cold or hot acid solution of the base treated with permanganate. Almost 50 % of the weight of the original dihydrochloride may be recovered as guanidine

glycine. The main results of the oxidation appear to be representable as follows:



In an oxidation experiment with standardized bromine water 83% of the oxygen required by the above expression was actually used, and in a similar determination with permanganate in hot solution, about 93%. The deficiency is readily explicable by the appearance of glycine originating in the manner suggested below.

Further evidence in support of the formulation adopted is concerned with certain observations on the alkaline hydrolysis of the base. These comprise the isolation of an intermediate product, apparently the corresponding carbamido-glyoxaline, and secondly the identification of glycine as an end-product of the hydrolysis of both the guanidino- and carbamido-compounds. The apparent significance of glycine as a matter of evidence will first be considered.

Purines, without substituents in the 7, 8 or 9 positions, on drastic hydrolysis with HCl, yield glycine, along with ammonia, CO₂ and sometimes CO and formic acid. Thus Schmidt [1883] showed that xanthine when heated for 6 hours at 180–200° with conc. HCl left glycine, and Krüger [1892] showed its presence in similar hydrolysates from adenine and from hypoxanthine. Its production from guanine under the less drastic conditions reported in the previous paper is thus not unexpected. When the purine carries a methyl substituent in position 7 glycine is replaced in the hydrolysate by sarcosine, as shown in the case of theobromine by Schmidt & Pressler [1883], and in the case of caffeine by Rosengarten & Strecker [1871]. The latter, however, hydrolyzed the caffeine with hot saturated barium hydroxide until ammonia was no longer evolved.

Fargher [1920] isolated glycine in an attempt to prepare 4-(or 5)-aminoglyoxaline by reduction of the corresponding nitro derivative. Fargher supposed the aminoglyoxaline to be unstable and transformed into 4-(or 5)-glyoxalone, which readily disintegrates at the 1 : 2 and 3 : 4 positions with the production

oxalate by the addition of slight excess of bromine and keeping the solution at room temperature for a few hours, for the separation of the small doubly-pointed needle-like crystals. They burn without melting. Boiling water or dilute acid has no apparent effect on the material. A little of the substance suspended in water readily dissolves on addition of a small amount of NaOH. Curiously enough the solid is insoluble in cold 2.5 N NaOH. If the slightly alkaline solution is acidified, the material quickly recrystallizes. If however the alkaline solution is kept for some time at room temperature, or boiled and quickly cooled, and made acid, then no separation occurs. Guanidine is then readily precipitated from the solution by picric acid, oxalic acid by Ca, and in presence of excess H₂SO₄ the solution avidly absorbs permanganate. Determination by this means indicates about 60% oxalic acid in the substance in question. Guanidine is present to the extent of about 40% of the substance according to measurement by the Tiegs method. Nessler's test for ammonia is negative, and indeed the total N of the substance is accounted for by the guanidine content. Micro-analysis of the substance (Dr Stantial) gave: C, 26.82, 26.56; H, 4.18, 4.10; N, 29.82, 29.64%. C₆H₁₂O₄N₆ requires C, 25.70; H, 4.32; N, 30.01%. The values are not very good for C₆H₁₂O₄N₆, representing a complex of equal proportions of guanidine oxalate (CH₅N₃·C₂H₂O₄) and oxalyl guanidine (CH₅N₃·C₂H₂O₃). There is good evidence that the substance contains only guanidine and oxalic acid, but attempts to prepare it from these constituents have so far failed. No substance of a like composition appears to have been described, and the product obviously calls for further study.

of glycine. From 4-nitro-5-methylglyoxaline Fargher similarly obtained α -alanine.

Glyoxaline, and glyoxalines with alkyl substituents or a saturated side-chain, e.g. histidine, are exceedingly resistant to oxidizing or hydrolytic agents. They do not absorb permanganate and may be heated for long periods with strong acid or base without appreciable change.

The stability of the glyoxaline ring is apparently lost in such a substance as 4-(or 5-)aminoglyoxaline, in the substances to be described in this paper, and presumably as it exists in the purine nucleus. The writer has observed that a small amount of urocanic acid treated in cold acid solution with permanganate readily yields a positive ninhydrin test, most likely attributable to glycine; which suggests that the presence of an unsaturated aliphatic side-chain leads to lability of the glyoxaline nucleus.

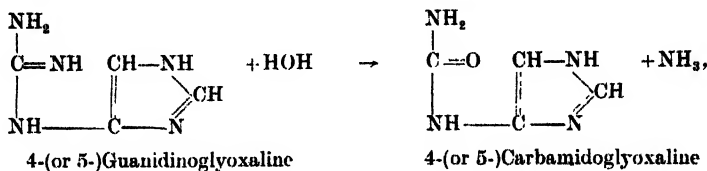
It thus appears at the outset that the likelihood of obtaining a stable glyoxaline from a structure such as 4-(or 5-)guanidinoglyoxaline is slight. But, on the other hand, from the foregoing discussion, the formation of glycine by hydrolysis with acid or base or by oxidation with permanganate of the substance in question would seem to be strong evidence for the presence in it of the glyoxaline nucleus.

From $\text{Ba}(\text{OH})_2$ hydrolysates of the base, glycine has been isolated and identified as its copper salt and as glycine sulphate. Experiments have not so far been specially designed to obtain a maximum yield of glycine (most probably exhaustive hydrolysis with $\text{Ba}(\text{OH})_2$ or HCl) but about 30% of the theoretical yield has been obtained as copper glycine. Under the conditions no more was to be expected, so that little doubt can be entertained about its quantitative significance.

The mechanism of the hydrolysis of the base has not been fully elucidated. On the analogy of arginine it was expected that baryta hydrolysis would yield urea and 4-(or 5-)aminoglyoxaline. The picrate obtained from the hydrolysate had a much lower M.P. than guanidinoglyoxaline dipicrate, but it was soon discovered to be a mixture of this with the picrate of a different substance. The guanidinoglyoxaline dipicrate was less soluble in hot water than the unknown picrate and a mixture, estimated from this property roughly as two parts of the unknown picrate to one part of the known, was readily obtained, M.P. 185–190°. The further separation was extremely tedious as the two picrates had like solubilities at ordinary temperature, and further fractional crystallization appeared impossible. The mixture, with as large a proportion of the unknown picrate as was readily attainable, was freed of picric acid in the presence of HCl by ether and evaporated to a small volume. On the addition of a suitable amount of absolute ethyl alcohol, rhombic or square-shaped crystals appeared. These were readily distinguishable under the microscope from the needles or spars formed by guanidinoglyoxaline dihydrochloride, which was always present in the filtrate from such crystals. The filtrate was re-treated with picric acid and the procedure repeated. About 0.5 g. of a hydrochloride was thus obtained from about 2.5 g. of guanidinoglyoxaline sulphate. After hydrolysis about 25% of the material is recoverable unchanged, and it appears that part of the compound here sought decomposes further to glycine and ammonia. With the application of less tentative methods a better yield may be attainable.

I have again to thank Dr Stantial, Toronto, for the C and H values in the following analysis of the substance thus prepared. (Found: C, 29.36, 29.72; H, 4.34, 4.23; N, 34.61, 34.42; Cl, 21.80, 21.76%. $\text{C}_4\text{H}_6\text{ON}_4 \cdot \text{HCl}$ requires C, 29.54; H, 4.34; N, 34.46; Cl, 21.80%.)

With the diazo-reagents the substance yields an exquisite red colour which shows only a tinge of blue in presence of slight excess of NaOH. After oxidation with permanganate there is no trace of guanidine according to Tiegs's test. A positive ninhydrin test, almost certainly attributable to glycine, is obtained on hydrolysis of the substance with Ba(OH)₂. The relationship of the substance to the guanidinoglyoxaline would thus appear to be correctly represented by



and on the arginine analogy, the citrulline analogue has been produced.

The changes intermediate between the carbamidoglyoxaline and glycine are still obscure, but may yield to further study. The Ba(OH)₂ hydrolysates from both the guanidino- and carbamido-compounds are characterized by a number of colour tests suggesting the presence of 4-(or 5-)aminoglyoxaline [Fargher & Pyman, 1919], or perhaps of 4-(or 5-)glyoxalone. Of these a strongly marked sodium nitroprusside test would appear to suggest the presence of a linkage of the type HC=C.OH. It appears unlikely that aminoglyoxaline would yield a positive nitroprusside test. Furthermore, the hydrolysate in alkaline solution acquires a blue colour on exposure to air. If a little of the acidified hydrolysate is treated with a drop of NaNO₂ solution, and the mixture added to a NaOH solution of β-naphthol a brown colour is produced. Fargher and Pyman [1919] attribute a similar reaction to diazotization of an NH₂ group on the benzenoid 4- or 5-C atom of the glyoxaline nucleus. The last test which may be mentioned is the production of a grass-green colour with ninhydrin. The solution shows an absorption band at about 6460 Å., and its colour is quite unlike that obtained in the usual amino-acid test. Whether all of the tests here mentioned are attributable to one or more substances can only be decided by further investigation.

A number of colour tests for the two new bases will be described in the experimental section. These are both highly reactive substances, and often respond in such an extraordinary manner that certain of such tests seem to merit detailed consideration. Some of the tests, not already mentioned, have a bearing on the constitution of the substances.

EXPERIMENTAL.

Preparation of salts and free base.

4-(or 5-)Guanidinoglyoxaline dihydrochloride. The preparation and analysis of this salt are reported in the preceding paper. Recrystallization is best carried out by dissolving in a minimum amount of water, if necessary boiling with charcoal and filtering, evaporation *in vacuo* to copious crystal formation, release of vacuum and heating the tube over a flame to dissolve the crystals, followed by the gradual addition of absolute alcohol with shaking. Two to three volumes of absolute alcohol suffice for the separation of 80–90% of the material present. The crystals are fine needles or spars according to the rate at which they are formed. After washing with absolute alcohol and drying at 110°, they have the composition C₄H₅N₅·2HCl. m.p. 288° (decomp.) with quick heating; darkening begins about 270°. The substance is readily soluble in water.

4-(or 5)-Guanidinoglyoxaline sulphate. This is readily prepared from the dihydrochloride by the addition of the theoretical amount of 5 *N* H_2SO_4 and drying in a vacuum desiccator in presence of NaOH ; e.g. 2.0 g. $\text{C}_4\text{H}_7\text{N}_5 \cdot 2\text{HCl}$ were treated as described, and the residue was dissolved in 5–6 ml. water. This was concentrated *in vacuo* to 2–3 ml. and 2–3 vol. absolute alcohol were added. When the tube was cooled and scratched with a glass rod crystallization quickly set in. (There is always a tendency for the sulphate, even in pure form, to separate first as an oil on addition of excess alcohol, but scratching the tube invariably initiates crystal formation.) The tube was left in the refrigerator overnight, the crystals collected, washed with absolute alcohol and dried in the air for some hours; yield 2.282 g. Dried at 110° to constant weight, 1.972 g. Loss of 0.310 g. H_2O or 13.6%. (Calculated for $\text{C}_4\text{H}_7\text{N}_5 \cdot \text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$, 13.9% H_2O .) The crystals are square or rhomboid, m.p. 219° .

4-(or 5)-Guanidinoglyoxaline dipicrate. When picric acid is added even to dilute aqueous solutions of either of the above salts an insoluble precipitate is quickly formed. When the picrate is dissolved in a minimum of hot water and allowed to cool slowly long heavy spars with uneven edges and golden colour appear. There are frequently also fused masses of short stout prisms with well-defined crystal faces; m.p. $216\text{--}217^\circ$. Found by the nitron method of Busch & Blume [1908] 77.0 and 77.2% picric acid. (Calculated for $\text{C}_4\text{H}_7\text{N}_5 \cdot 2\text{C}_6\text{H}_3\text{O}_7\text{N}_3$, 78.5% picric acid.)

Guanidinoglyoxaline. 0.3 g. guanidinoglyoxaline sulphate was dissolved in a little water, and titrated in an atmosphere of N with 0.5 *N* $\text{Ba}(\text{OH})_2$ until the SO_4 was removed. The solution was centrifuged in the stoppered tube and the supernatant fluid transferred to a small basin, which was quickly placed in a desiccator and left to dry *in vacuo* in presence of solid NaOH and H_2SO_4 . At this stage a drop of the aqueous solution on a tile was slightly alkaline to Porrier's blue, indicating *pH* near 11.0. After drying for a day the material in the basin showed no sign of crystallization, but remained as a thick gum, readily thinned by warming.

Guanidinoglyoxaline carbonate. The free base described above was dissolved in a little warm absolute alcohol and CO_2 led through the solution. The solution remained alkaline to litmus and showed no sign of crystal formation. An excess of dry ether was finally added; a clear oil separated. After the stoppered tube had been kept for a few hours definite crystal formation took place at points on the sides, but the main body of oil was only semi-solid after keeping 2 days in the refrigerator. Some was removed to a watch glass, but it remained sticky and apparently hygroscopic. On placing the watch glass in an oven at about 110° , the substance quickly melted and did not resolidify on cooling. On the addition of a drop of conc. HCl there was vigorous effervescence with loss of CO_2 . The addition of a little alcohol to the watch glass was soon followed by the formation of the typical spars of the dihydrochloride over its whole surface. A little of the carbonate tested on a tile with thymol blue had a *pH* near 8.5.

Oxidation with bromine.

20.1 mg. $\text{C}_4\text{H}_7\text{N}_5 \cdot 2\text{HCl}$ titrated with 0.112 *N* Br required 3.0 ml. for slight excess in the cold. After keeping for a short time the excess Br disappeared and guanidine oxalate separated. When the solution was made slightly alkaline with NaOH the precipitate dissolved. It was brought to the boil, cooled and made up to 10 ml. With Tiegs's reagents and a colour standard of pure guanidine hydrochloride the solution was found to contain 5.43 mg. guanidine from the possible 5.96 mg., or a yield of 91%.

3.0 ml. 0.112 *N* Br yield 2.69 mg. O. 20.1 mg. $C_4H_7N_5 \cdot 2HCl$ using 2 atoms of O require 3.25 mg.; the O utilized is 82.8% of theoretical amount.

By nesslerization of a similar solution it was found that about 80% of the non-guanidine-N was present as ammonia in the solution. At least part of the remainder was present as glycine as shown by the ninhydrin test.

The solution prepared as above and acidified with acetic acid precipitates calcium oxalate on the addition of $CaCl_2$ solution. The precipitate dissolves in excess H_2SO_4 , and the resulting solution absorbs permanganate.

When the solution prepared as above, is acidified with H_2SO_4 , and distilled *in vacuo* into dilute NaOH solution, the distillate readily reduces permanganate in alkaline solution in the cold, indicating the presence of formic acid.

Oxidation with $KMnO_4$.

54.5 mg. $C_4H_7N_5 \cdot H_2SO_4$ were treated with 5 drops 5 *N* H_2SO_4 and 10 ml. 0.1 *N* $KMnO_4$. When the solution was heated to boiling, a copious brown precipitate, mixed with a white precipitate of guanidine oxalate, persisted. The solution was made alkaline with 2.5 *N* NaOH and treated with excess H_2O_2 . MnO_2 was filtered off, the solution made slightly acid to litmus and treated with excess picric acid; wt. of picrate 53.2 mg. (theory 70.4 mg. guanidine picrate); yield 75.7%.

In a similar manner, but adding $KMnO_4$ until the purple colour persisted, 54.1 mg. $C_4H_7N_5 \cdot H_2SO_4$ gave 52.7 mg. guanidine picrate (theory 69.9 mg.); yield 75.4%.

The picrates thus obtained were mixed and recrystallised without change of m.p. (324–327°, according to rate of heating). 9.8 mg. of the picrate gave 18.0 mg. nitron picrate, or 7.62 mg. picric acid, 77.75%. 8.8 mg. gave 16.9 nitron picrate, or 7.15 mg. picric acid, 81.3%. (Calculated for guanidine picrate 79.5% picric acid.)

50.9 mg. $C_4H_7N_5 \cdot H_2SO_4$ titrated in hot solution in presence of excess H_2SO_4 showed a persistent brown end-point after 8.5 ml. 0.1 *N* $KMnO_4$ had been added. This is the equivalent of an uptake of 6.8 mg. O. 50.9 mg. of the salt require on the basis of 2 atoms 7.3 mg. O. Oxygen used is thus 93.2% of theoretical amount.

Oxalic acid, ammonia, formic acid and glycine can be shown to be present in the solution by the means adopted above for the material oxidized with bromine.

Hydrolysis with $Ba(OH)_2$.

Preparation of 4-(or 5-)carbamidoglyoxalinehydrochloride. 0.97 g. $C_4H_7N_5 \cdot H_2SO_4$ was placed in a small flask with a reflux condenser, on top of which was fixed a Folin bulb containing standard acid. A tube was fused through the side of the flask, leading close to the bottom, so that nitrogen could be bubbled through the mixture during hydrolysis. There were placed in the flask 3.5 g. $Ba(OH)_2$ and 30 ml. water, giving a medium approximately *N* for hydrolysis. A slow current of N was admitted and the flask boiled with a micro-burner for 4.5 hours. It was allowed to cool while the stream of N flushed the remaining NH_3 into the standard acid. The flask was detached from the condenser and 6.0 ml. 5 *N* H_2SO_4 added, at which point the reaction was slightly acid to Congo red. The $BaSO_4$ was removed, and the filtrate evaporated to about 10 ml. *in vacuo* in a boiling-tube. About 1 g. of picric acid, dissolved in a minimum quantity of boiling water was then added to the solution. After cooling the picrate was collected, washed with water and absolute alcohol and dried; yield 0.99 g.; m.p. 176°. (The filtrate

was kept.) It was transferred to a 50 ml. centrifuge-tube, 20 ml. water were added, and the mixture was boiled. It was centrifuged hot and the supernatant liquid poured into another tube. The residue was re-extracted with 3 ml. water in the same fashion and the supernatant fluid added to the first. From the combined extracts 0.626 g., M.P. 184°, was recovered. The residue was dissolved in a minimum amount of boiling water (23 ml.), then allowed to crystallize; yield 0.252 g.; M.P. 217°, the same as the dipicrate of guanidinoglyoxaline.

At this stage the 0.626 g. picrate was combined with similar material giving in all 1.98 g. It was put in a separating funnel with about 50 ml. water and 2 ml. conc. HCl and the picric acid removed with ether. The resulting solution was concentrated *in vacuo* to about 20 ml., boiled with a little charcoal and the filtrate concentrated to about 3 ml. About 2 vol. absolute alcohol were added and crystals soon separated. After keeping for 1–2 hours in the refrigerator the mixture was filtered, washed with absolute alcohol and dried; yield 0.539 g.; M.P. 204°. The filtrate was kept for re-treatment with picric acid.

The 0.539 g. was boiled in a little water with charcoal, the filtrate evaporated to about 4 ml. and 2 vol. of absolute alcohol were added. There was a slow appearance of large flat plates, appearing sometimes in clusters on edge as spars; yield 172 mg.; M.P. 210°, with yellow melt. By adding an equal vol. of alcohol to the filtrate a further crop of 50 mg. of identical material was obtained. By evaporation of the filtrate and again adding alcohol a further amount of 146 mg. of material of M.P. 206° was obtained. This crop was contaminated with guanidinoglyoxaline dihydrochloride, and with its filtrate it was re-treated with that above, with picric acid. By repeating the whole procedure a further crop of 59 mg. of pure carbamidoglyoxaline hydrochloride was obtained. The three crops with M.P. 210° were combined and recrystallized without change in M.P. or behaviour towards the diazo-reagents. When dried their composition was represented by $C_4H_6ON_4 \cdot HCl$.

Carbamidoglyoxaline sulphate. 31 mg. of the hydrochloride were treated in a small basin with 0.19 ml. *N* H_2SO_4 and dried in a vacuum desiccator. The crystal mass was dissolved in about 0.5 ml. water and filtered through a micro-funnel into a small tube. The volume was reduced *in vacuo* to about 0.1 ml. when signs of crystallization appeared. A few drops of absolute alcohol were added and the tube placed in the refrigerator. The filtered, air-dried crystals weighed 29.624 mg. After heating to constant wt. at 110° they lost 2.651 mg. or 8.95% H_2O . (Calculated for $(C_4H_6ON_4)_2 \cdot H_2SO_4 \cdot 2H_2O$, 9.33% H_2O .) M.P. of dry sulphate is 197° with slow decomposition and foaming. (Found: S, 9.05%. Calculated for $(C_4H_6ON_4)_2 \cdot H_2SO_4$, S, 9.15%.)

Carbamidoglyoxaline. 22.73 mg. of the sulphate were dissolved in about 2 ml. water and 0.26 ml. 0.5 *N* $Ba(OH)_2$ was added, so that the solution was neutral to litmus and free from Ba and SO_4 ions. It was filtered and evaporated to a small drop, and a small amount of a mixture of equal parts absolute alcohol and ether added. A slight pinkish colour appeared in the solution. The crystals, wt. 4 mg., formed broad spars; M.P. 194–195° with golden melt. They were just perceptibly alkaline to sensitive litmus, and to bromothymol blue, so that carbamidoglyoxaline is apparently only feebly basic. (Found: N, 40.87% on somewhat impure material. $C_4H_6ON_4$ requires N 44.41%.)

Carbamidoglyoxaline picrate. To about 20 mg. of the hydrochloride an excess of picric acid was added. The precipitate was washed with absolute alcohol, wt. 46 mg.; M.P. 210°. The material was recrystallized from 2 ml. water; beautiful iridescent needles and spars formed on slow cooling, of a much more homogeneous appearance than is obtained with guanidinoglyoxaline dipicrate;

M.P. 210°. 11.624 mg. air-dried material, on heating to constant wt. at 110°, lost 0.554 mg. or 4.77 %. (Calculated for $C_4H_6ON_4 \cdot C_6H_5O_7N_3$, H_2O , 4.83 % H_2O .) By nitron method, found 61.9 and 62.2 % picric acid. (Calculated for $C_4H_6ON_4 \cdot C_6H_5O_7N_3$, 64.5 % picric acid.)

Note on the $Ba(OH)_2$ hydrolysis.

By titration of the contents of the Folin bulb it was found that 100 mg. N had been lost as NH_3 during the 4-5 hours' hydrolysis of 0.97 g. $C_4H_7N_5 \cdot H_2SO_4 = 0.5437$ g. $C_4H_7N_5$. (Total N 304 mg.) Tests for urea on such hydrolysates have invariably been negative. It has been recorded that approximately 1 g. of a mixed picrate was obtained from 0.97 g. of the original salt. A rough computation would indicate that this is composed of about 0.6 g. guanidinoglyoxaline dipicrate and 0.4 g. carbamidoglyoxaline picrate. This means that 0.130 g. of the original base remained unchanged, leaving 0.4137 g. taking part in the hydrolysis. Of the latter 0.142 g. is accounted for by formation of the carbamido compound, thus leaving approximately 0.27 g. to be accounted for. Under the conditions of the hydrolysis it may thus be concluded that about 25 % of the original guanidinoglyoxaline remains unchanged, 25 % is converted into carbamidoglyoxaline, and 50 % of the material is present as product(s) not yet recognized. Guanidine may be present in such hydrolysates but only in traces, and the bulk of the NH_3 obtained by titration probably arises from this side-chain. There is also little glycine present in the freshly prepared hydrolysate.

Isolation of glycine from $Ba(OH)_2$ hydrolysates.

Filtrates from the picrates, as obtained under the preparation of carbamidoglyoxalino hydrochloride, were collected from a number of batches representing about 3.0 g. $C_4H_7N_5 \cdot H_2SO_4$, with a view to the study of the substance giving a positive nitroprusside test. After keeping for 2-3 weeks in the refrigerator it was observed that this test had become faint, but that the ninhydrin test had become very marked. The combined picric acid filtrates were accordingly evaporated to about 10 ml. and the excess picric acid, along with a little more of the mixed picrates, was filtered off. Picric acid was removed from the solution with ether and the aqueous residue treated with Ag and $Ba(OH)_2$. Only the filtrate from the latter was kept. It was freed from Ag by H_2S , concentrated, made neutral to litmus and boiled with $CuCO_3$: wt. of Cu salt 200 mg. (= 140 mg. glycine). It was dissolved in 2 ml. N H_2SO_4 and saturated with H_2S . On evaporating to about 1 ml. and adding excess absolute alcohol, there were obtained 136 mg. of typical glycine sulphate, M.P. 225°.

According to the computation above only about half of the starting material is available for glycine formation in such hydrolysates; in this case about 1.5 g. $C_4H_7N_5 \cdot H_2SO_4$, or 840 mg. $C_4H_7N_5$, which could theoretically yield 504 mg. glycine. The actual yield of 140 mg. is thus 28 % of theory. It may be observed that several of the batches in the combined picric acid filtrates were subjected to milder hydrolysis than that detailed, and an appreciable amount of material, which may have produced glycine on further hydrolysis, was discarded in the Ag- $Ba(OH)_2$ precipitate.

Oxidation of 4-(or 5)-carbamidoglyoxaline.

10.4 mg. $C_4H_6ON_4 \cdot HCl$ were treated with acid permanganate as described for the guanidino-compound. The resulting solution gave a negative diazo-test, a negative Tiegs test, but a strongly positive ninhydrin test. Urea could not be detected in the solution.

Ba(OH)₂ hydrolysis of 4-(or 5-)carbamidoglyoxaline.

7.5 mg. $C_4H_6ON_4 \cdot HCl$ were heated with 2 ml. $N Ba(OH)_2$ in a small side-arm tube for 4½ hours. 0.95 mg. N was evolved as NH_3 . The solution was neutralized with 2 ml. $N H_2SO_4$ and the $BaSO_4$ separated; the filtrate gave a positive nitroprusside test, a slowly developing purple colour with $NaOH$ and, after diazotization, a brown colour with β -naphthol.

On treating a little of the solution with an equal vol. of 1 % ninhydrin and a drop of pyridine a greenish yellow colour appeared immediately, and with very slight heat a grass green colour quickly developed. On prolonging the heating, the unstable green disappeared and was replaced by the blue colour typical of glycine. From these tests it is apparent that the carbamido-compound is an intermediate in the formation of the unknown substance(s) discussed above as arising from 4-(or 5-)guanidinoglyoxaline. It may well also be an intermediate in the formation of glycine.

General properties and colour tests of the two bases.

Both bases and their salts above mentioned, with the exception of the picrates, are readily soluble in water. Both bases, but especially guanidinoglyoxaline, are soluble in absolute alcohol. Their salts are insoluble in alcohol and in most organic solvents. The salts of guanidinoglyoxaline are colourless, those of the carbamido-compound tend to have a faint pink tinge. The picrates of course are yellow and not markedly different in colour from guanidine picrate. Both bases are precipitable by phosphotungstic acid, salts of mercury and by silver in presence of $Ba(OH)_2$.

Diazo-test. When a drop of a dilute solution of the guanidino-compound is added to a mixture of 1.25 ml. 1.1 % Na_2CO_3 and 0.5 ml. Koessler and Hanke reagent, a dirty yellowish green may be momentarily observed followed immediately by a bright methylene blue-like colour. If more than about 0.1 mg. of the substance has been added the blue colour persists for some time, but if the colour is faint at the outset it quickly fades. Under the latter circumstances it can be stabilized for some time by the addition, quickly after coupling, of a few drops of 2.5 $N NaOH$. If the blue-coloured alkaline solution is made acid, it changes to a garnet-like red.

When a drop of a dilute solution of the carbamido-compound is similarly added to the mixed diazo-reagent, a momentary yellow colour is first perceptible followed by the immediate formation of a brilliant red colour. In dilute solution this fades but again may be stabilized by the addition of a few drops of dilute $NaOH$, with little tendency towards blue. When large excess of strong alkali is added, however, the colour is changed to a deep pure blue. In acid solution the colour is again red.

With either substance the test is very sensitive. The addition of as little as 0.1 γ of either of the hydrochlorides to the above amount of reagent is sufficient to give a perceptible colour.

Sakaguchi test. (According to the modification of Weber [1930, see also Poller, 1926].) When about 0.05 mg. guanidinoglyoxaline dihydrochloride in 0.5 ml. water is treated with 1 drop of 2.5 $N NaOH$ and 1 drop of 0.02 % α -naphthol solution, the tube cooled in ice, and 1 drop of sodium hypobromite solution (1 g. Br in 50 ml. 5 % $NaOH$) added, followed quickly by a few drops of 40 % urea solution, a bright green colour, rapidly changing to blue and slowly fading to purple and brown, appears.

An equal quantity of the carbamido-compound similarly tested gives a pink colour which fades rapidly.

Both tests are abnormal. The carbamido-compound, as stated above, contains no guanidine yet gives a test characteristic of monosubstituted guanidines according to Poller. Weber mentions that tyrosine, histidine and tryptophan interfere with the test. I have found that tyrosine gives no colour, histidine some colour, and tryptophan a marked red colour with the test. With urocanic acid a brilliant pinkish red colour, which quickly fades, is produced. So, apparently, the Sakaguchi test is not entirely specific for substituted guanidines. With the guanidino-compound the normal red is replaced by a blue, perhaps by an influence similar to that at work in the diazo-test.

Tiegs's test. When about 0.05 mg. of the guanidino-compound in 0.5 ml. water, to which has been added a drop of 2.5 *N* NaOH, is mixed with 0.3 ml. Tiegs's reagent, a clear deep green colour, gradually changing to blue, is produced. When a somewhat greater amount of the carbamido-compound is similarly tested a dirty greenish brown colour appears.

Both tests are again abnormal. The behaviour of the guanidino-compound in this test is analogous to its behaviour in the diazo- and Sakaguchi tests. The behaviour of the carbamido-compound is here quite anomalous.

Behaviour with Cu. A neutral solution of either substance when heated with CuCO_3 yields a blue solution, and a black precipitate is formed. A very dilute solution of the guanidino-compound added to Benedict's solution leads to the formation of a greyish purple precipitate. If shaken with a little freshly prepared $\text{Cu}(\text{OH})_2$, with subsequent addition of a solution of Na_2CO_3 , a similar salt is formed in the cold. On heating, the salt blackens. The Cu salt is soluble in excess of strong alkali and does not appear in Fehling's solution, but when the latter is heated a deeper blue or violet colour appears. The carbamido-compound yields no precipitate with cold Benedict's solution, but a black precipitate appears on heating.

Urea tests. (a) *Ehrlich's.* A greenish yellow colour is obtained when a 1% solution of carbamidoglyoxaline hydrochloride is treated with a little *p*-dimethylaminobenzaldehyde. On heating, a clear pink colour is produced—an anomalous behaviour. The guanidino-compound shows a negative test.

(b) *Schiff's.* The carbamido-compound responds like allantoin to this test, the colour being more blue than that given by urea. The guanidino-compound shows a negative test.

Neither substance is precipitable by xanthidrol or affected by urease.

Ninhydrin test. Both substances yield an apparently identical buff-red colour when a little of their salts in solution is heated with an equal volume of 1% ninhydrin in presence of a drop of pyridine.

Folin's uric acid test. When 1 drop of the Folin & Marenzi [1929] reagent is added to a little solution of either base, and made alkaline with Na_2CO_3 , or a slight excess of NaOH, a blue colour is produced. A rough estimate indicated that 1 part of guanidinoglyoxalino dihydrochloride gave the same amount of colour as 2.83 parts of carbamidoglyoxaline hydrochloride or 2 parts of uric acid. The *biuret test* is positive for the carbamido-compound and negative for the guanidino-compound.

With the *murexide test* the guanidino-compound leaves a lemon-yellow residue made slightly orange with alkali, whilst the carbamido-compound leaves a blackish residue which goes quite black with either NaOH or NH_4OH .

The guanidino-compound gives a negative *xanthoproteic test*, the carbamido-compound gives a deep reddish colour on addition of ammonium hydroxide.

The *Jaffé test* is positive for both substances.

The following tests are negative for both substances: *Millon*, *Adamkiewicz*, *Mörner*, *Molisch*, *Obermeyer* and *nitroprusside*.

When 10 *N* NaOH is brought in contact with a dry guanidinoglyoxaline salt a bright pinkish red colour appears and then rapidly fades.

A solution containing 10·13 % of guanidinoglyoxaline dihydrochloride in a 2 dm. tube when examined in a Bellingham and Stanley polarimeter with the green Hg line showed no optical activity.

I should like to acknowledge the great help given me throughout this work by my colleague, Dr M. M. Cantor. The Carnegie Research Grant Committee of the University has assisted financially.

NOTE ON THE DISSOCIATION AND ULTRAVIOLET ABSORPTION SPECTRA.

BY WILLIAM DOUGLAS MCFARLANE.

Guanidine is a base of the strength of potassium hydroxide. It will be seen (Fig. 1) that guanidinoglyoxaline is also a relatively strong base. The influence of

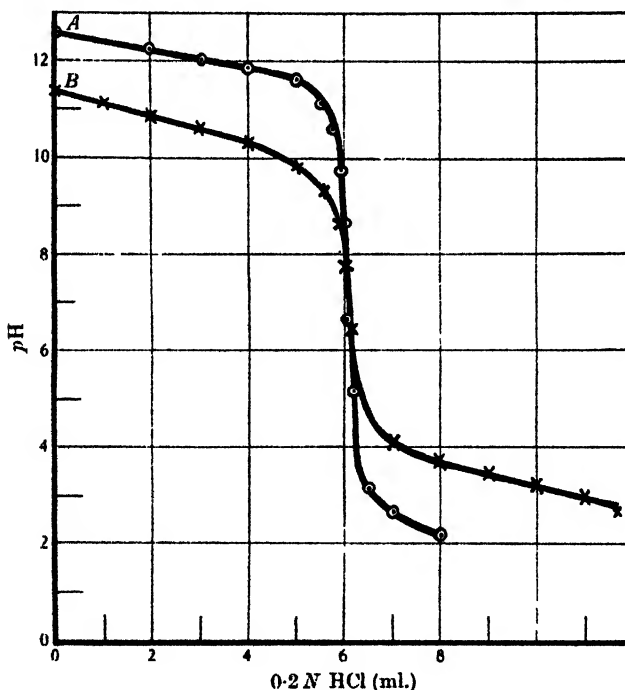


Fig. 1. Titration curves of (A) guanidine hydrochloride and (B) guanidinoglyoxaline dihydrochloride.

salt concentration on the dissociation of these highly ionized bases may, for present purposes, be considered as negligible so that the ionic strength may be

determined directly from the titration curves. The dissociation constant of guanidinoglyoxaline is at approximately pK_H 10.5 while, according to Hall & Sprinkler [1932], that of phenylguanidine is at pK_H 10.77. The introduction of an unsaturated negative substituent, such as a phenyl or glyoxaline group reduces, therefore, the basicity of the parent compound. Davis & Elderfield [1932] have shown that on the other hand a saturated positive substituent such as piperidine has no such effect.

Ring compounds of the unsaturated pyrimidine series exhibit, according to Heyroth & Loofbourow [1934], a marked degree of selective absorption. The compounds examined in this study (Fig. 2) showed end absorption typical of

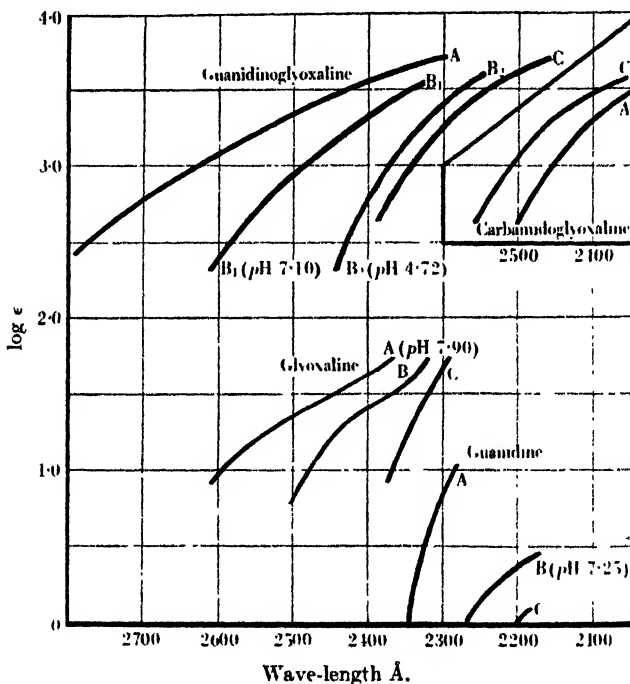


Fig. 2. Absorption curves of 0.1 *M* guanidine hydrochloride; 0.02 *M* glyoxaline; 0.000287 *M* carbamidoglyoxaline monohydrochloride and 0.000174 *M* guanidinoglyoxaline dihydrochloride in various solvents: A, 0.1 *N* NaOH; B, phosphate buffer; C, 0.1 *N* HCl.

glyoxaline itself. It is well known that the absorption curves of most organic acids and bases change in some regular way with the pH of the solution, owing to the difference in the absorption exhibited by the anions, cations or undissociated molecules. In this regard the compounds examined are no exception. Previous measurements on glyoxaline and guanidine have been made by others without taking this into consideration. The shift in ultra-violet absorption by guanidinoglyoxaline at varying pH is typical of a highly ionized base whilst the behaviours of glyoxaline and carbamidoglyoxaline are characteristic of weakly ionized compounds.

The following figures indicate the relative absorption intensities of these compounds in 0.1 *N* NaOH.

	Molecular extinction coefficient at wave- length 2300 Å.
Guanidinoglyoxaline	5680
Carbamidoglyoxaline	5600
Glyoxaline	300
Guanidine	90

EXPERIMENTAL.

1. *Titration curves.*

The data represented in Fig. 1 were obtained as follows. Solutions of the free base, approximately 0.033 *M*, were prepared by dissolving 119.4 mg. guanidinoglyoxaline dihydrochloride, or 246.1 mg. guanidinoglyoxaline dihydrochloride in distilled water; adding NaOH to neutralize the HCl exactly and diluting to 50 ml. with distilled water. A hydrogen half-cell of the Hildebrand type and a saturated calomel half-cell were used for the pH measurements made during the titration of the two solutions with 0.2 *N* HCl.

These measurements have been repeated using the procedure of Davis and Elderfield [1932] in which the solution of the free base is prepared by adding the required amount of Ba(OH)₂ to a solution of the sulphate. The results obtained by the two methods were identical, within the limits of accuracy of the technique.

2. *Absorption curves.*

To obtain solvents which were completely transparent down to the limit 2160 Å. of the photographic plates employed, it was found necessary to recrystallize the primary and secondary phosphates used in the preparation of the phosphate mixtures, and to make the NaOH solution from NaOH prepared from sodium. The pH of the buffered solutions was determined by the quinhydrone electrode.

The absorption spectra were determined with a Bellingham and Stanley quartz spectrograph fitted with a rotating sector photometer. The wave-length measurements were made with a plate-measuring instrument of the same manufacture. The sector readings gave directly the value of the logarithm of the transmission *T*. From this the molecular extinction coefficient (ϵ) was obtained by Beer's law

$$I = I_0 10^{-\epsilon cl},$$

where *c* is the concentration in g. mol. per litre and *l* is the thickness (in this case 1.0 cm.) of the absorbing solution.

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CLXIX. CHANGES IN GROWTH AND WATER CONTENT OF THE BONES OF NEWLY BORN PUPS AND KITTENS.

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HAMMETT [1925] concluded that "The percentage of water in bones of both sexes decreases with age. This is largely due to ash deposition. The increase in percentage of organic matter is a minor factor." He also pointed out, "If the percentage values be charted it is seen that the curves for ash and water simulate that of a monomolecular autocatalyzed reaction." Bone growth and ossification are however the results of numerous changes taking place simultaneously. A study of these individual changes in normal bone growth was undertaken before the study of certain pathological conditions. The methods used have been described [Burns & Henderson, 1935]. Cats and dogs were used, as they were available and of suitable size. Some work was also done on the herbivores, goats, sheep and rabbits, but as they showed the presence of certain species differences they will be dealt with in a subsequent paper.

The cats and dogs used were bred domestically and the mothers were given ample mixed domestic diets. The mother cats received $\frac{1}{2}$ pint of milk per head per day normally: this was increased to $\frac{1}{2}$ pint during lactation. The bitch received $\frac{1}{2}$ pint usually and 1 pint during lactation. The animals were free to play in the sunshine. Ample calcium and phosphate were added to the diet in the form of bones and fish heads. The young weaned themselves gradually, and were put on to the same diet as their mothers with the necessary addition to the quantity of milk. Weaning was complete at 21–25 days. Figures were also secured from two adult cats and one adult dog, of unknown breed and diet, and from pooled samples from the long bones of the hind-legs of four embryos from a large mongrel bitch. The age of the embryos was unknown, but they were hairless and the fresh weight of the sixteen femora and humeri was 0.819 g. which may be compared with 1.463 g., the weight of the two femora of a newly born pup from a much smaller terrier bitch.

The first factor studied in these animals was the relation between age and the relative proportion of epiphysis to whole bone both fresh and dry (Table I).

Table I. *Epiphysis as % of total femur.*

	Kittens														
	Litter 1			Litter 2			Litter 3*			Litter 7*			Puppies		
	0	3	16	7	11	17	45	160	0	3	10	1	24	60	160
Age (days) ...	0	3	16	7	11	17	45	160	0	3	10	1	24	60	160
% fresh weight	40.0	40.8	43.3	47.9	46.8	38.0	34.6	29.9	39.8	39.1	45.4	44.8	41.3	44.0	—
% dry weight	23.1	30.4	28.0	30.2	30.0	22.2	27.7	27.9	22.1	17.2	27.2	22.4	24.2	37.4	29.0
Ca % dry	—	—	—	1.22	1.11	1.68	9.5	15.6	—	—	—	0.88	3.99	9.6	—
epiphysis															0.43

* Same mother as litter 2.

In litter 1 which was studied from birth, there was an increase in the weight of both fresh and dry epiphysis relative to the whole bone in the first 3 days of extra-uterine life. In litter 7, these increases occurred between the 3rd and 10th days. Thereafter there was a steady decrease in the percentage of the fresh bone attributable to epiphysis. At an age of about 20 days, however, the proportion of the weight of the dry bone due to epiphysis began to increase as the epiphysis ossified. (This ossification is indicated in Table I by the calcium content of the dry epiphysis.) On the other hand, in the puppies, no significant change took place in the first 60 days in the proportion of the epiphysis in the fresh bone. In the dry bone, the proportion of the weight due to epiphysis increased with ossification and then fell with the continued growth of the shaft. In the embryos, the epiphysis formed a much larger proportion of either wet or dry bone than at any stage after birth. In the case of the kittens, two animals from another litter from the same mother as litters 2 and 3 gave figures at 23 days which agreed satisfactorily with those shown in the table. Two kittens 50 days old, however, from a cat of larger breed had relatively much heavier and larger epiphyses than those shown in the table, indicating that breed influences relative rates of growth.

Femur, humerus and tibia were all examined from each animal and all changed in the same way. The figures for the humerus and femur in the kittens only differed significantly in the 160-day animal in which the epiphysis formed 35.2 and 32.2% of the wet and dry humerus respectively as against 29.9 and 27.9% in the femur. The epiphysis of the tibia at all stages after the first few days of life was smaller in proportion to the bone than were those of the other two. In the puppies, the same general relations were found.

Water and fat contents of epiphysis, diaphysis and whole bone at different ages were next studied (Table II). The fat content was negligible in the first few weeks of life. In the three litters of kittens shown in Table II, and one other (litter 4) in which only the whole bone was examined, there was a definite rise in water content of the whole bone in the neonatal period. In litter 7, this rise occurred between the 3rd and 10th days. After this the water content fell steadily. The initial increase was mainly due to an increase in the water of the diaphysis during this period, whilst the subsequent fall was due to the decrease in the proportion of fresh epiphysis to whole bone and to the decreasing water content of the epiphysis. After the first rise the water of the diaphysis remained roughly constant up to 50 days of age, whilst the water of the epiphysis fell from 79.5 to 68.5%. Estimations on animals from other litters all accorded with those shown.

No consistent and significant difference in the water contents of femur and humerus was observed as reported by Hammett [1925] for rats. Out of 11 animals in which femur and humerus were compared, the femur contained more water than the humerus in 10, but differences averaged 2% and never exceeded 3%. When femur and tibia were compared, the femur again contained more water in 10 cases, and the differences ranged from 1 to 5% and averaged 2%. When diaphyses were compared instead of bone, the femur again generally contained more water than either humerus or tibia (average difference 3%), and in two cases differences as high as 10% were noted.

In the puppies the diaphyseal water content rose steadily from 1 to 60 days of age, whilst the epiphyseal water content decreased. For the whole bone, the water content was roughly constant over this period. It is possible that the maximum point found in the kittens was missed in the puppies because of the few samples available. In the embryos, the water contents of epiphysis and

Table II.

Kittens												
Age (days)	Litter 1			Litter 7*			Litter 2*			Litter 3*		
	<1	3	16	<1	3	10	7	11	17	45	Cat	Pups
Bone	59.3	70.0	69.4	66.0	58.7	67.8	64.8	68.1	63.6	60.5	36.4†	Embryos
Bone	—	—	0.4	—	—	—	1.0	0.8	1.7	0.8	—	72.0
Epiphysis	79.4	81.6	80.2	81.1	81.8	80.8	77.9	79.5	78.8	68.5	—	82.1
Epiphysis	—	—	0.7	—	—	—	0.7	0.5	0.3	0.7	—	—
Diaphysis	45.9	58.4	61.2	56.0	43.9	57.2	52.7	58.0	54.4	56.3	—	50.4
Diaphysis	0.0	0.2	0.7	—	—	—	1.3	0.7	2.5	0.9	—	—

* Litters from same cat.

† Fat and water.

‡ Litters from same dog.

Table III.

Kittens												
Age (days)	Litter 1			Litter 7			Litter 4			Pups		
	<1	3	7	<1	3	10	1	8	25	33	60	60
Water femur %	59.3	70.0	69.4	66.0*	58.7*	67.8*	61.0	64.9	65.7	61.5	63.2	63.2
Width of femur mm.	—	—	—	—	—	—	3	3	4	5	2.5	6.0
Width of cavity mm.	Pin point	Cortex	—	Pin point	Much wider	—	1	1.5	3	3.5	Pin point	4.5
Texture of cortex	Very brittle	Very brittle	Brittle	Tibia hard	Femur hard	Mod. hard	Very hard	Hard	Brittle	Brittle	Very hard	Hard
				Femur brittle	Tibia less hard	—	—	—	—	—	—	—

* Femur and tibia.

diaphysis did not differ much from those of the bones of the newly born pups, but owing to the large proportion of epiphysis to whole bone, the water content of the whole bone was relatively higher.

Differences between femur, tibia and humerus in the pups were of the same order as in the kittens, but the femur in these animals did not so consistently contain more water, the differences being fairly evenly distributed in the two directions.

Table III shows that these changes in the water content of the bone are associated with changes in the growth processes and in the texture of the bone.

The newly born kitten from litter 1, three 24-hour old kittens from litters 4 and 5, and the newly born pup had bones which were almost solid in the middle of the shaft and were extremely hard and difficult to cut but did not splinter. In two 3-day kittens (litter 6), and the 8-day animal from litter 4 the bone cortex was fairly thick and hard but the marrow cavity was definitely wider. On the other hand, in the 3-day-old animal from litter 1, although the bone was wider than that of the newly born litter-mate, the cortex was of paper thickness and so brittle that it fell to pieces when cut. All the bones of litter 2 from 7 days upwards showed well-marked marrow cavities, and their cortices were quite brittle. From Table III it may be seen that the widths of bone and marrow cavity were such that the 8-day bone (litter 4) would lie inside the marrow cavity of its 25-day litter-mate. The latter again would almost lie inside the cavity of its 33-day old litter-mate. Similarly the bone of the newly born pup would lie inside the 24-day bone, and this again almost inside the bone of the 60-day pup. The increase in the water of the diaphysis, and the consequent increase in or maintenance of the water content of the bone was thus mainly associated with the widening of the marrow cavity, which was accompanied by rapid destruction of bone. Between 0 and 3 days in litter 1, 8 and 25 days in litter 4, and between 1 and 24 days and again between 24 and 60 days in the pups, the entire cortex was eaten away and a new cortex built up. During this period of rapid growth the texture of the cortex underwent a marked change and became very brittle and splintered readily. The changes in the texture of the cortex and in the water content and width of marrow cavity did not exactly coincide, e.g. in the 8-day animal of litter 4, a fairly wide cavity and high water content were found in bones with fairly hard cortex. In both pups and kittens the cortex remained brittle and splintered readily when cut, until its increasing thickness made cutting with an ordinary knife impossible. This stage was reached between the ages of 60 and 160 days. The nature of the changes determining the alteration in the cortex of the bone is being studied histologically.

The question of whether the solidity of the bones at about the time of birth represents a store of calcium laid up during the stages of slow growth at the end of the intra-uterine period, or whether it merely marks a stage of bone development, is being examined by the study of those animals in which the bones of the new-born are at different stages of ossification. The results of these studies will be communicated shortly. The fact however that one 3-day-old tibia was more solid than that of its new-born litter-mate suggests that the beginning of rapid bone breakdown does not necessarily coincide with birth.

The slight increase in the water content of the epiphysis between 0 and 3 days of age (litter 1, Table II), was accompanied by an increase of 25 % in the weight of the epiphysis, whilst the diaphysis increased by 10 % in length, but lost by about 10 % in weight. In litter 2, both epiphysis and diaphysis increased by 100 % in weight between 7 and 11 days, but the epiphysis made no further gain between 11 and 17 days, whilst the diaphysis increased by a further 50 %. In

litter 7 on the other hand, the bones of the new-born and 3-day animals were equal in weight and length, and the fresh epiphysis formed the same proportion of the fresh bones in each, but the diaphysis of the 3-day-old bone contained much more solid (organic and inorganic) than that of the new-born. Bone growth is thus represented by a large number of simultaneous reactions whereby organic tissue is laid down and ossified in certain places, while both organic and inorganic materials are removed from others.

Hammett [1925] found consistent differences in the rates of growth in the femur and humerus of rats.

There was no consistent difference in the rates of growth of femur, humerus and tibia in puppies and kittens, although the tibia at all ages was slightly smaller than either of the other two (Table IV).

Table IV. *Weight in g.*

Age (days) ...	Kittens					Pups			
	7	11	17	45	160	1	24	60	160
F.	0.714	1.496	1.628	4.886	18.70	1.463	9.164	20.10	48.9
H.	0.890	1.506	1.831	4.674	16.84	1.901	9.696	19.03	47.8
T.	0.510	1.013	1.282	3.839	16.71	0.906	5.855	14.74	41.4

It seems possible that the differences which he noted in water content were associated with the differences in rates of growth.

SUMMARY.

1. The water content of very young bone is determined mainly by the size and water content of the epiphysis and of the marrow cavity.

2. Shortly after birth in pups and kittens there is an increase in the water content of the long bones, due mainly to an increase in the water content of the diaphysis.

3. During this period, there is a rapid increase in the size of the marrow cavity, and a change in the texture of the bone cortex.

4. These changes are found in femora, humeri and tibiae. There is no consistent difference in water content between these bones.

The expenses of this research were defrayed from a grant to Prof. Burns by the Medical Research Council to whom we desire to express our thanks.

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CLXX. THE MINERAL CONSTITUENTS OF BONE.

II. THE INFLUENCE OF AGE ON THE MINERAL CONSTITUENTS OF BONES FROM KITTENS AND PUPS.

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METHODS of bone analysis and sampling have been described in a previous paper [Burns & Henderson, 1935]. In this investigation changes in calcium, phosphate and carbonate of bone from kittens and pups were studied in relation to age. Dry fat-free calcified cartilage, cancellous bone and samples of cortex from the middle of the shaft and from the growing ends of the diaphyses were used.

Table I. *Calcium and carbonate in the cortex from the middle of the shaft (cats).*

Age (days)	...	0-6	7-13	14-20	21-48	49-84	160	Adult*	Adult*
No. of samples...		9	8	6	12	9	1	1	1
Ca %:									
Range		17.8-22.9	16.3-20.6	19.0-20.6	18.7-22.2	20.7-22.9	—	—	—
Mean		20.9	18.5	19.8	20.7	21.9	23.3	24.0	25.5
Standard deviation σ		1.5	1.37	0.60	—	—	—	—	—
CO ₂ as % Ca:									
Range		9.4-12.3	9.5-11.0	8.6-9.6	9.0-10.7	9.5-10.7	—	—	—
Mean		10.6	10.0	9.1	10.0	10.0	10.9	11.5	12.9
σ		0.92	0.53	0.12	—	—	—	—	—

* Unknown breed.

From Table I it may be seen that the mean value for the calcium content of the dry fat-free cortex of the middle of the shaft of the femur fell slightly between the first and second weeks of post-natal life, and then rose very slowly over several months. When the carbonate content is expressed as that percentage of the calcium with which it can combine, it is evident that the carbonate also fell and then rose over the same periods, but that the carbonate fell more markedly than the calcium, so that its proportion to the latter also decreased. This decrease in the proportion of carbonate to calcium was moreover continued into the third week, and although the difference between the figures for the first and second weeks is of doubtful significance, there seems to be probably a real difference between the first and third weeks. After 21 days the proportion of carbonate to calcium slowly rose to reach its highest level in the adult animals. The change in calcium content and in the carbonate content relative to the calcium was also found in the pups (Table III).

The standard deviations for the calcium values for the groups 0-6 days, 7-13 days (Table I), are such that the difference between the means is not

statistically significant, but if the bones are re-grouped according to the texture of the cortex rather than age [Burns & Henderson, 1936], then the 3-day bone (Ca 17.8%) falls into the second group and the 8-day bone (Ca 20.6%) falls into the first group, and the means become 21.2 and 18.1%, with standard deviations 1.07 and 1.12 respectively. Three pairs of samples of bone from litter-mates of the same age (two at 23 days, and one at 50 days) had calcium contents which agreed within 2%, but a pair from day-old animals and another at 3 days gave figures which differed by 5-6%. In these two pairs however the difference in calcium content was again accompanied by differences in size of bone and texture of cortex, the harder cortex or smaller bone (i.e. the bone in which post-natal change was least advanced) being in each case associated with the higher calcium content.

In view of the following facts it seems probable that the rapid post-natal growth and remodelling of the bone results in a decrease of the calcium content even of the cortex in the middle of the shaft: (1) In litter 1 when the change in bone texture was marked between birth and 3 days the Ca content fell from 20.7 to 17.8% in this period. (2) In litter 7, between 3 and 10 days the content fell from 22.9 to 19.8%. (3) In litter 4 and the pups the changes in bone texture between 1 and 8 and 1 and 24 days respectively were accompanied by a drop in Ca of 4 and 3% (changes not significant in themselves but consistent in direction). (4) The difference in the Ca of the first two groups is just significant when they are classed by texture and not by age.

In nine animals femur and humerus were both examined, and differences in calcium content ranged from -5 to +8% with an average of +2.2% (the humerus being to this extent more calcified than the femur). In seven pairs of femora and tibiae the femora were more calcified than the tibiae by an average of 2.5% (range of difference -8 to +6.5%). There was thus no evidence of significant differences in the rates of calcification of the different bones of kittens and pups as found by Hammett [1925] for rats.

From the calcium, carbonate and phosphate of the bone was calculated the ratio of calcium combined with phosphate, called by Howland *et al.* [1926] the residual Ca/P (here called R (Ca)/P).

For forty-six samples of cortex from the middle of the shaft this ratio did not vary with age; twenty-three figures lay between 1.88 and 1.93, fifteen between 1.82 and 1.88, and eight between 1.74 and 1.82. No figure below 1.84 was found in animals over 45 days old, and the adults showed ratios of 1.88-1.91. For the dogs, the ratio ranged from 1.81 to 1.92, the sample from the adult animal and two samples from 60-day pups showing 1.88 and 1.90.

Table II. *Calcium and carbonate in the cortex from the ends of the diaphyses (dogs).*

Age (days)	...	0-6	7-13	14-20	21-49	160	Adult	Adult
No. of samples	...	2	6	6	8	2	2	2
Ca %:								
Range		16.8 and 17.8	16.0-17.8	17.8-19.6	17.2-19.0	18.6 and 19.4	20.7 and 21.7	23.1 and 24.9
Mean		17.3	16.9	18.5	18.1	19.0	21.2	24.0
σ		—	0.58	0.57	—	—	—	—
CO ₂ as % Ca:								
Range		9.6 and 10.1	8.2-9.0	7.7-8.9	7.7-9.3	9.1 and 9.3	11.3 and 11.6	13.4 and 13.2
Mean		9.8	8.6	8.5	8.4	9.2	11.4	13.3

From Table II it may be concluded that the calcium content of the cortex from the growing ends of the diaphysis remained low throughout the growth period and then slowly increased approximately to that of the cortex from the

middle of the shaft in the adult animals. There was a slight increase just significant (i.e. difference exceeding 2σ) after the period of rapid post-natal growth. Throughout the growth period, the proportion of calcium combined with carbonate was always less in the cortex from the diaphyseal ends than in that from the middle of the shaft. The early period of growth was again accompanied by a drop in the carbonate relative to the calcium. In the adult the ratio of carbonate to calcium also approximated to that found in the cortex from the middle. In six animals in which femur, humerus and tibia were examined, the calcium of the cortex from the ends of the femur exceeded that of the humerus by an average of 2% and that of the tibia by an average of 5%, but differences equalling these were found between the two ends of the same bone, and no significant difference between the bones could be demonstrated.

Table III. *Calcium and carbonate in the cortex of pups' bones.*

	Middle					Embryos			Ends				
Age (days) ...	<1	24	60	160*	Adult†	Middle	Ends	<1	24	60	160*	Adult†	
Ca %	21.7	20.9	22.4	24.5	25.8	16.9	12.8	17.2	18.4	19.6	17.7	23.3	
CO ₂ as % Ca	12.7	11.2	11.4	13.0	13.9	6.1	4.7	11.1	9.8	10.8	9.7	12.3	

* 2nd litter.

† Unknown breed.

The changes in the cortex from the ends of the diaphyses in the pup (Table III) were similar to the changes in the kitten, except that at 160 days both calcium and carbonate were relatively low. This was possibly a sampling error, since there was such a marked difference in shape between the long shaft and relatively small epiphysis of the 160-day bone and the much shorter bone at 60 days, that it was difficult to determine the proportion of the cortex to take for analysis.

R (Ca)/P was determined for twenty-eight samples of this tissue from cats and eight samples from dogs. Four figures from two adult cats ranged from 1.87 to 1.91. Of the remaining twenty-four, three lay between 1.88 and 1.95, fifteen between 1.82 and 1.88 and six between 1.74 and 1.82. These in no way varied with age. The figure for the adult dog was 1.87 and for the seven growing animals ranged from 1.81 to 1.91 without relation to age.

In the neo-natal period the cancellous tissue showed more marked changes in calcium content with age than did the cortex (Tables IV and V).

Table IV. *Calcium and carbonate contents of cancellous bone in cats.*

Age (days) ...	0-6	7-13	13-20	21-49	50-84	160	Adult	Adult
No. of samples ...	11	6	9	16	18	1	1	1
Ca %:								
Range	17.8-23.63	13.8-17.9	14.7-16.9	12.5-17.6	12.0-16.8	—	—	—
Mean	20.96	15.8	16.1	14.7	14.4	14.5	16.1	17.4
σ	1.5	1.2	0.71	—	—	—	—	—
CO ₂ as % Ca:								
Range	9.3-11.7	8.2-10.2	7.5-9.4	6.7-9.6	7.7-10.0	—	—	—
Mean	10.3	9.1	8.4	8.4	8.9	8.3	10.3	12.6

The mean value for the calcium content of this tissue in the kittens fell between the first and second weeks from 20.9 to 15.8%, a quite significant drop, the lowest value in the first week being found in the brittle 3-day bone, whilst the highest value in the second group was found in the hard 8-day bone. From the second week there was a further slight drop followed later by a rise, but the

figure for the adult animals did not exceed some figures found at all stages of the growth period. On the other hand whilst the initial drop was also found in the pups (Table V), the 160-day-old pup and the adult dog both showed significant increases in calcium content. It is not possible to decide whether this is a species difference, or whether the 160-day-old pup was better able to utilize the bones in the diet than the 160-day-old kitten.

Table V. *Calcium and carbonate content of cancellous bone and calcified cartilage in dogs.*

Age (days) ...	Cancellous bone					Embryos		Calcified cartilage			
	<1	24	60	160	Adult	Can- cellous	Calcified cartilage	<1	24	60	160
Mean Ca %	22.2	18.7	17.3	22.1	22.1	15.7	8.5	20.3	17.4	19.7	19.2
CO ₂ as % Ca	11.4	10.5	9.3	10.2	14.0	4.4	3.6	10.4	8.4	9.2	9.0

In both dogs and cats, the initial fall in the calcium of the cancellous bone was accompanied by an even greater fall in the carbonate content, and the carbonate remained low relative to the calcium until growth ceased, when it again rose towards the figure for adult bone as found in the cortex of the fully grown animal.

Differences up to 15% were found between the calcium contents of the cancellous bone from the two ends of the same bone, and in the seven kittens in which femur, humerus and tibia were examined no differences exceeding this were found between the different bones, the average difference being of the same order as for the cortex.

Sixty-two determinations of phosphate were made on the tissue from cats and R (Ca)/P for the two adults was 1.87. Nine figures ranging from 1.88 to 1.94 were found at ages varying from 3 to 50 days. The other figures ranged from 1.68 to 1.87 and averaged 1.81, thirteen falling below 1.80. Out of the nine figures for dogs, four fell between 1.88 and 1.92, and others ranged from 1.81 to 1.87. The 160-day-old animal gave 1.87 and the adult 1.92.

The calcium and carbonate contents of the calcified cartilage from kittens of different ages are shown in Table VI and of pups in Table V.

Table VI. *Calcium and carbonate contents of the calcified cartilage of kittens.*

Age (days) ...	0-6	7-13	14-20	20-48	49-84	160
No. of samples ...	8	5	6	14	17	1
Ca %:						
Range	15.6-20.2	17.1-18.6	16.0-18.4	17.1-19.8	14.6-18.7	—
Mean	18.1	17.9	17.5	17.9	17.2	17.2
CO ₂ as % Ca:						
Range	6.5-9.9	6.7-7.5	6.1-7.0	6.2-8.6	7.2-9.5	—
Mean	8.5	7.1	6.6	7.4	8.1	7.3

The figures for the one litter of pups (Table V) suggested that the post-natal drop in calcium was also to be found in the calcified cartilage but the more numerous figures for the kittens did not support this. In these animals the mean calcium remained roughly constant throughout the growth period. The post-natal drop in the proportion of carbonate to calcium was however found. In the pups this proportion was much the same as in the other rapidly growing tissues, i.e. the cancellous bone and the cortex from the ends of the diaphyses, but in the kittens it was consistently lower. In the kittens 50 estimations of R (Ca)/P were

made and 14 as high as 1.88 occurred at 1, 8, 11, 50 and 84 days. The other thirty-six figures lay between 1.71 and 1.87 and averaged 1.82. In the pups the figures ranged from 1.84 to 1.88 and averaged 1.85.

In the embryos, the calcium content and the proportion of carbonate to calcium in all the tissues were lower than the lowest figures found after birth, but again the proportion of carbonate was highest in the cortex from the middle of the shaft and lowest in the calcified cartilage. The figures for R (Ca)/P however, 1.76, 1.82, 1.85 and 1.88, were similar to figures from post-natal samples.

As was found previously for water, the results of the analyses of bones of kittens for calcium failed to indicate the systemic differences noted by Hammett [1925] for rats, so a femur and humerus from each of two animals were examined for water and ash by his methods, whilst the other femur and humerus were examined for Ca by the methods used in this investigation.

Table VII.

Kitten 1.				Ca %	
	Wt. g	Water %	Ash %	Cortex	Cancellous
F.	1.250	65.8	14.08	20.15	16.83
H.	1.388	65.3	14.91	21.07	17.63
Kitten 2.					
F.	1.402	65.9	13.89	20.6	16.85
H.	1.468	66.1	14.57	20.8	16.64

Water differences (Table VII) lay within the accuracy of the estimation (an accuracy determined mainly by the speed with which the bones were cleaned and weighed). The ash of the humerus exceeded that of the femur by 6 and 4 %, and the calcium of the dry cortex from the middle of the shaft of the humerus exceeded that of the femur by 4 and 1 % respectively.

It seems possible that the difference in ash content between the two bones of the rat may be associated with the different rates of growth of the bones in that animal. In the more slowly growing humerus, the more heavily calcified portions of the bone would form a greater proportion of the whole than in the more rapidly growing femur. In kittens this difference in the rate of growth is negligible, and differences in rate of ash deposition are also small.

Bauer *et al.* [1929] noticed a diminution in the number of trabeculae in kitten bones shortly after birth. They concluded from this that calcium is stored in the trabeculae and that this calcium store is drawn on during the period of rapid post-natal growth. From the change in calcium content in the different parts of the bone, and the complete destruction and rebuilding of the cortex which takes place during this period, it seems however that the trabeculae do not differ from cortical bone in this respect, at this stage. The proportion of cancellous bone to total bone in the young kittens decreased from 17 % in the new-born to 9 % at 16 days, but in view of the difficulty of securing all the cancellous bone the significance of these figures is uncertain. Any theory explaining the processes of ossification must take into account bone destruction as well as bone growth. Since both appear to take place side by side in the same bone, it seems that bone growth cannot be wholly controlled by changes in the systemic blood supply, but must be largely determined by purely local factors and that, at any rate as long as growth continues, bone is in dynamic equilibrium with its tissue fluids.

Consideration of the values for R (Ca)/P shows that out of the 186 samples from the bones of kittens, 138 (i.e. 76 %) gave ratios ranging from 1.82 to 1.95,

and averaged 1.87. Hammett [1925] found an amount of magnesium in the bones of rats equivalent to about 2-3% of the calcium. A few estimations of magnesium were made in the course of this investigation, by colorimetric determination as phosphate after precipitation of calcium. Magnesium figures varied from 0.35 to 0.49% with corresponding calcium 16.3 to 24.4%. It was found that figures for R (Ca)/P 1.91, 1.78, 1.77, 1.84 and 1.86, became when corrected for magnesium 1.97, 1.85, 1.84, 1.90 and 1.92 and a total Ca/P (CO_2 not determined) was increased from 1.91 to 1.97. If therefore an average of 0.06 is added to R (Ca)/P as found to correct for magnesium, it is found that 138 out of the 186 lie between 1.88 and 2.01, and of these 87 lie between 1.91 and 1.97. Only 13 lie between 1.98 and 2.01, whilst 38 are between 1.88 and 1.91. This is roughly the distribution to be expected if the true figure is 1.94, the theoretical figure for tertiary calcium phosphate. There are however 48 figures (26% of the total) with ratios below 1.88, which seem to fall outside the range of experimental error. Shear & Washburn [1932-33] have criticized the use of micro-methods in the analysis of bone and have developed a much more accurate technique, but for this are needed amounts of material much in excess of those available in a detailed study of young growing bone. They point out that with examinations made by the less accurate methods "deviations from the theoretical composition (i.e. $3\text{Ca}_3(\text{PO}_4)_2\text{CaCO}_3$) have usually been ascribed either to shortcomings of the analytical methods employed, or to experimental error. It is not impossible, however, that these discrepancies may have been due to actual variations in the composition of bone". The forty-eight samples in this study with corrected R (Ca)/P of less than 1.88 all occurred in bones from animals less than 60 days old, and they occurred most frequently in the most actively growing tissues; e.g. of the total samples they formed 35% in the cancellous bone, 25% in the calcified cartilage, 21% in the cortex from the ends of the shaft and 17% in the cortex from the middle of the shaft. This suggests that the actual period of rapid growth or destruction of bone may be marked by the presence in the bone of phosphate other than the tertiary calcium or magnesium salt. Shear & Kramer [1928] have adduced evidence to show that in ossification the secondary salt CaHPO_4 may be first deposited. The corrected ratios 1.74-1.87 found in the forty-eight samples would be compatible with a mixture of 10-20% of the secondary salt and 80-90% of the tertiary salt. Roseberry *et al.* [1931] showed (1) that there were none of the characteristic lines of crystalline CaHPO_4 in the X-ray spectrogram of fresh young bone, and (2) that a mixture of such bone and crystalline CaHPO_4 in the ratio 10:1 (i.e. bone salt: CaHPO_4 approximately 3:1) gave such lines quite clearly. They considered that if the secondary salt occurs, it is present in smaller proportion than this. It is not clear however whether their method would detect CaHPO_4 if it formed less than 25% of the total salt. Since only a few samples of growing bone could contain as much as this (from R (Ca)/P) and samples from different parts of the same bone may differ considerably, it is clear that the detection of such small quantities of the secondary salt would be difficult.

The small amounts of non-tertiary phosphate indicated may moreover only be soluble phosphates accumulated locally during bone growth or destruction and would not therefore be detected by the physical methods. If such phosphate were not combined with calcium, it would only constitute from 2 to 6% of the total, and its presence could only be finally demonstrated by its isolation. Almost all the samples examined for phosphate were analysed for both total and inorganic phosphate. No definite evidence of the presence of organically combined phosphate was found in the trichloroacetic acid filtrate.

Roseberry *et al.* [1931] also showed that (1) the main crystalline compound in the bone salt belonged to the apatite group and was very similar to dahlite ($2\text{Ca}_3(\text{PO}_4)_2\text{CaCO}_3$); (2) that bone salt contained no calcite (crystalline CaCO_3); (3) that tertiary calcium phosphate is a crystalline member of the apatite series. Kramer & Shear [1928] have already shown that old bone (diaphysis) contains more carbonate in proportion to calcium than does young bone (metaphysis). The results presented here show that the carbonate content of bone is roughly proportional to the rate at which that particular portion of bone is being laid down and removed. The relative carbonate content of calcified cartilage is low at all ages, that of the cancellous tissue and the rapidly growing cortex slightly higher, while the highest figure is found in the more slowly growing cortex of the middle of the shaft. When growth at the ends of the shaft has ceased, the carbonate content of the whole cortex and of the cancellous bone, in dogs and cats, tends to approximate to the figure found in dahlite (i.e. 14.2% of the calcium combined with carbonate). Sendroy & Hastings [1926-27] showed that when serum and certain salt solutions were shaken with CaCO_3 , no calcium was precipitated, but when shaken with $\text{Ca}_3(\text{PO}_4)_2$ calcium carbonate was precipitated. If at some stage in the growth cycle of bone tertiary calcium phosphate were in contact with fluids containing carbonate it seems probable that the salt would react to form a carbonato-phosphate complex, and that, up to the point at which all the salt was converted into a dahlite-like compound, the longer the phosphate was in contact with the fluid, the more compound salt would be formed. In the cortex of the middle of the shaft, where, after the post-natal spurt in growth, growth and destruction take place relatively slowly, the phosphate remains in contact with the tissue fluids of the bone long enough to maintain a high and increasing proportion of carbonate. In the rapidly removed calcified cartilage, on the other hand, the carbonate remains relatively low.

The very low carbonate contents of the bone tissues from the embryo pups suggest that the insoluble bone salt may be originally deposited as phosphate without the presence of any carbonato-complex. Further work is being carried out on embryos to test this point. But, within limits, the carbonate content of a bony tissue does seem to be a measure of the age of the tissue, and therefore a measure of bone growth in the region of the bone from which the tissue was taken.

SUMMARY.

1. Immediately after birth in pups and kittens there is a decrease in salt content of the diaphysis. This decrease is most marked in the cancellous tissue. This period must be avoided in any experiments designed to test a decalcifying or recalcifying agent.

2. Later the calcium content of the cortex rises slowly, but in the cancellous bone it displays irregularity and remains low in the kitten, while rising slowly in the pup.

3. In kittens, the difference between femur and humerus is very slight, and can probably be explained by the very slightly slower rate of growth in the latter.

4. In temporary tissues such as calcified cartilage, the carbonate is always low relative to the calcium, but its proportion increases in the tissues which are destroyed more slowly.

5. In the bone of the adult cat and dog, the proportion of carbonate to calcium approaches that of dahlite.

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CLXXI. A NOTE ON THE REVERSIBLE ENZYMIC OXIDATION OF *d*-GLUCO-ASCORBIC ACID.

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It was shown some time ago [Zilva, 1934] that the apple contained an enzyme resembling the "hexoxidase" described by Szent-Györgyi [1931] which could dehydrogenate directly or indirectly *l*-ascorbic acid. Tauber *et al.* [1935] have since demonstrated the presence of this or a similar enzyme in Hubbard squash (*Cucurbita maxima*) and there seems reason to believe that it is fairly widespread in the plant kingdom. An experiment is here described which shows that the enzyme present in the apple is also capable of dehydrogenating another member of the ascorbic acid series of compounds, *d*-gluco-ascorbic acid (*d*-3-ketoglucoheptonofuranolactone) which unlike *l*-ascorbic acid is not antiscorbutically active even in high doses [Zilva, 1935]. The experiment therefore suggests that the enzymic dehydrogenation of these compounds takes place independently of their stereochemical structure and of their biological activity.

10 mg. of *d*-gluco-ascorbic acid were dissolved in 30 ml. of distilled water. To 25 ml. of this solution 5 ml. of expressed apple (Bramley's Seedling) juice previously tested on *l*-ascorbic acid for the presence of the enzyme were added. The solution was adjusted to pH 4.4 and kept at room temperature. Aliquot portions were removed at various intervals and titrated with *N*/1000 indophenol. 5 ml. of the solution which decolorized 13 ml. of the indicator immediately after the addition of the enzyme reduced 6.3 ml. after 95 min., 2.7 ml. after 195 min. At the end of 295 min. it failed to decolorize any indophenol. The solution was then reduced with hydrogen sulphide as described by the writer in previous communications. After the hydrogen sulphide had been displaced by nitrogen for seven and a half hours, 5 ml. of the solution decolorized 13.7 ml. *N*/1000 indophenol. This figure dropped only to 13.2 ml. after 26 hours' displacement. The *d*-gluco-ascorbic acid was therefore completely dehydrogenated by the enzyme. The amount of the compound present originally in 5 ml. should have reduced 13.8 ml. of *N*/1000 indophenol.

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CLXXII. THE IDENTITY OF THE INDOPHENOL-REDUCING SUBSTANCE IN THE JENSEN RAT SARCOMA.

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(Received 28 May 1936.)

A NUMBER of recent investigations carried out in this laboratory offered the opportunity of adapting the biological and spectrographic procedures to the co-ordination of the *l*-ascorbic acid content with the indophenol-reducing capacity of certain animal tissues. As conflicting views exist concerning the identity of the reducing substance or substances present in tumours (for historical details see following paper [Boyland, 1936]), it was considered by us desirable to utilize this technique in attempting to obtain more precise information. This was made possible by the kindness of Dr E. Boyland of the Research Institute of the Royal Cancer Hospital who generously consented to supply us with the great quantity of material necessary for the investigation. Our results seem to suggest that almost the entire if not all the indophenol-reducing capacity of the Jensen rat sarcoma is due to *l*-ascorbic acid.

TECHNIQUE.

The experimental material. We are indebted to Dr Boyland for the following details. Small fragments of healthy Jensen rat sarcoma tissue were implanted with a small trochar needle under the skin in the flank of young rats under 120 g. in weight. The rats employed were either of the Wistar strain or of the "Middlesex Hospital" strain of tumour-susceptible rats. When the tumours had grown to a suitable size (4–8 g.) the tumour-bearing animals were killed by a blow on the head. The abdominal skin was cut and the tumour removed with forceps without opening the peritoneum. The tumours were removed daily for the biological test and were dispatched immediately to the Lister Institute. Forty-eight tumours were prepared for this purpose.

The biological tests. Immediately on arrival at the Lister Institute all the necrotic tissue was completely removed from the tumours. The remaining material was first ground with half its weight of powdered glass, and 60–100 % of its weight of freshly glass-distilled water was then added to the mixture which was thoroughly ground again. The somewhat coloured and turbid aqueous extract was next separated from the solid tissue by centrifuging. In order to determine the indophenol-reducing capacity of the preparation with the greatest possible accuracy a part of the extract was precipitated with 3 volumes of 5 % trichloroacetic acid, centrifuged and the clear colourless supernatant fluid titrated with the indicator at pH 2.5. The figures thus obtained were in fair agreement with those recorded by us in the original extract before treatment with trichloroacetic acid. The aqueous preparation was then administered to the experimental guinea-pigs in doses, the indophenol-reducing capacity of which corresponded to

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0.25 and 0.5 mg. of *l*-ascorbic acid respectively. The administration of the freshly prepared doses, which was carried out daily, began after the animals had subsisted for ten days on a scorbutic diet. The time which elapsed between the removal of the tumour and the dosing of the extract was about 2 hours. A trichloroacetic acid extract of the aqueous preparation was made and titrated in most cases also at the end of the dosing, but no significant loss in the indophenol-reducing capacity of the extract was noted. The animals were killed by chloroform 20 days after the commencement of the dosing and the degree of scurvy if any was assessed at the post-mortem examination. Daily doses of 0.25 and 0.5 mg. of *l*-ascorbic acid were administered to control groups of guinea-pigs.

The spectrographic examination. An aqueous extract of the tissue was prepared as above except that when the indophenol-reducing capacity was not high a suitably smaller quantity of freshly glass-distilled water was used. This extract was then treated with 8.6 parts by volume of absolute alcohol and 0.4 part by volume of 2.5% CdCl_2 in 90% alcohol. After removing the precipitate by centrifuging, the centrifugate was further diluted with an equal volume of

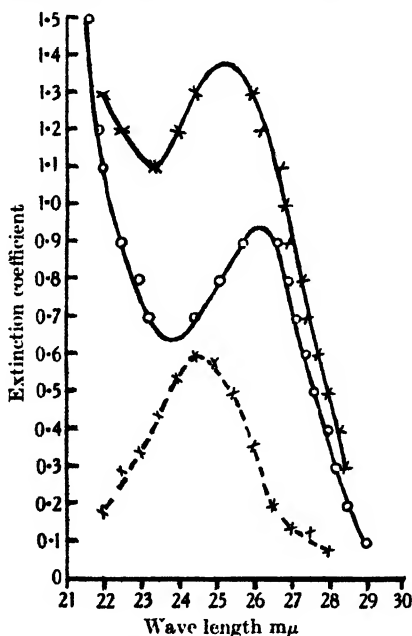


Fig. 1. Spectrographic results. The *l*-ascorbic acid content of this extract as calculated from the indophenol titration should have been 21.6 mg./100 ml. of extract. The above extinction coefficient corresponds to 21.5 mg./100 ml. of extract.

x—x Tumour extract. o—o Tumour extract after treatment with Cu.
x---x Ascorbic acid by difference.

absolute alcohol. One portion of this mixture (10 ml.) was then acidified with hydrochloric acid so as to give a final concentration of 0.02 *N* HCl and immediately compared in the spectrograph against a blank solution. To another 10 ml. of the above mixture 1 drop of 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution was added and the mixture allowed to remain for 2 hours. It was then acidified as before and examined against a control (see Fig. 1). This latter treatment destroys *l*-ascorbic acid entirely

when present in concentrations such as were encountered in these experiments, and consequently it could act as an index for the probable identity of the vitamin with the substance showing a point of maximum absorption at $245\text{ m}\mu$. Details of the spectrograph have already been given by us [Kellie & Zilva, 1936]. A 1 cm. absorption tube was used. The content of ascorbic acid was calculated from the extinction coefficient, since the concentrations were adjusted so as to fall within the limits in which Beer's law holds true [cf. Herbert *et al.* 1933]. The molecular extinction coefficient as determined by us for pure *l*-ascorbic acid in acid alcohol solution was found to be 10,000, and this value was therefore used in the calculation of the results.

RESULTS.

Fig. 2, which gives the graphic representation of the biological tests, shows that the responses of the animals on the *l*-ascorbic acid doses and of those which received the equivalent doses of tumour extract as assessed by indophenol titration are the same. From Table I we see further that the average of 15 spectrographic determinations accounts for 78.8% of the *l*-ascorbic acid as measured

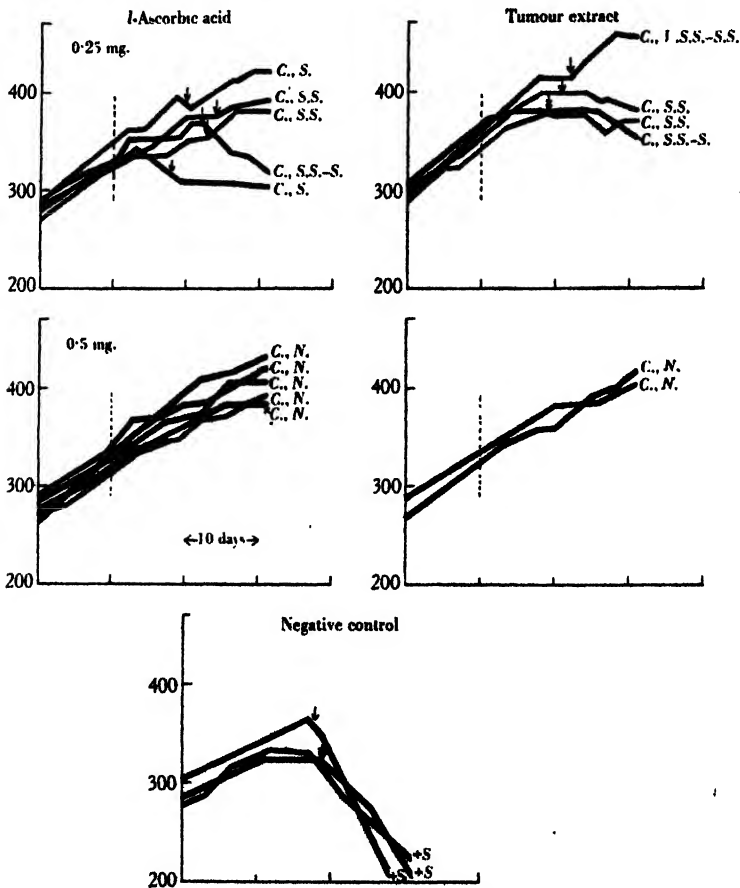


Fig. 2. Biological tests.

= beginning of dosing; ↓ = onset of clinical symptoms of scurvy; + = died; C. = chloroformed; N. = normal; V.S.S. = very slight scurvy; S.S. = slight scurvy; S. = scurvy.

Table I.

Ascorbic acid calculated from indophenol titration mg./100 ml. extract	Ascorbic acid found spectrographically mg./100 ml. extract	%
21.6	21.5	100
18.1	12.9	71
16.7	10.8	65
17.1	15.2	71
16.7	15.6	93
23.4	21.8	93
15.3	11.5	75
24.0	17.6	73
16.1 }	12.2 }	76 }
16.1 }	12.6 }	78 }
18.1	15.2	84
10.0	6.7	67
22.0	17.8	81
28.6 }	21.6 }	75 }
28.0 }	22.7 }	81 }
Average —	—	78.8

The bracketed figures refer to determinations carried out on the same extract within an hour of one another.

titrimetrically. That the characteristic absorption is due to *l*-ascorbic acid and not to chemically related compounds in this case is evident from the biological and indophenol tests. The average figures obtained by spectrographic measurements are, however, undoubtedly low. A difference of 21 % or even less would have been reflected in the condition of the animals, especially of those on the higher doses in the biological determination. It is also necessary to consider the fact in this connexion that figures tend to be lower than their real value in spectrophotometric assessments when concentrations of ascorbic acid in physiological fluids as low as those above are examined. This is illustrated by the experiment (Table II) in which plasma was submitted to the same precipitation treatment

Table II.

Ascorbic acid added mg./100 ml. plasma	Ascorbic acid found spectrographically mg./100 ml. plasma	%
24.9	22.4	90
29.4	24.6	84
30.3	29.0	96
24.9	22.8	92
Average —	—	90.5

with alcohol and CdCl_2 as the tumour extracts, and in which a known quantity of *l*-ascorbic acid of a similar order to the above was added to the final solution immediately before the spectrographic examination. An average of only 90.5 % of the added ascorbic acid is accounted for here. This error would be expected to be greater with tumour extracts in which the ascorbic acid is present before precipitation. It is of importance to note that in spite of the average of 78.8 % recorded by the spectrograph for these extracts, as high a figure as 100 % has been obtained (cf. Fig. 2), and that the average of the determinations in the case of tumours with a high content above 20 mg./100 ml. of extract, when the in-

herent error of the method would be expected to be less, was as high as 83·8%. These facts suggest that almost the entire indophenol-reducing capacity of aqueous extracts of Jensen rat sarcoma is due to *l*-ascorbic acid.

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CLXXIII. THE SELECTIVE ABSORPTION OF ASCORBIC ACID BY GUINEA-PIG TUMOUR TISSUE.

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BIERICH & ROSENBOHM [1933] were the first to show that the greater part of the material titratable by iodine in tumour extracts was not glutathione. They also showed that liver and brain tissue contained something other than glutathione which reduced iodine solutions; they called this substance "X". Boyland [1933] found that the amount of the X substance present in tissue was equivalent to the 2:6-dichloroindophenol-reducing power and so appeared to be ascorbic acid. A note, however, was added to the paper in proof saying that biological tests indicated the presence of some other reducing substance in Jensen rat sarcoma tissue. The results indicated that if the indophenol reduction were entirely due to ascorbic acid, actively growing tumour contained 0.4 mg. ascorbic acid per g. whilst the necrotic tumour tissue contained very little. These results have been confirmed by Edlbacher & Jung [1934], Watson & Mitolo [1934] and Woodward [1935]. Harris [1933], however, claimed as the result of biological tests, of which the full details have not been published, that only about one-third of the indophenol-reducing material of Jensen rat sarcoma was ascorbic acid.

Borghi & Deotto [1934] gave extracts of the Ehrlich mouse sarcoma to guinea-pigs on a scorbutic diet and the animals died with scurvy. Bierich & Rosenbohm [1934], however, found that aqueous extracts of JRS tumours had antiscorbutic activity, but their experiments were not sufficiently quantitative to prove conclusively that the total reducing power was due to ascorbic acid.

Watson [1936] has tested the antiscorbutic activity of dried tumour tissue. Unfortunately drying of the tissue destroyed about one-half of the reducing activity. In the case of JRS he found that the residual reducing value was equivalent to the antiscorbutic activity. Dried Dael and Biltris guinea-pig tumour, on the other hand, had practically no antiscorbutic power although it reduced 2:6-dichloroindophenol. The fresh guinea-pig tumour had only half as much ascorbic acid as the rat tumour and on drying about three-quarters of the reducing power was lost. Watson also found that the reducing power of a guinea-pig sarcoma fell to one-third its normal value if the animal were kept on a scorbutic diet, and in the case of one animal the reducing power increased to the normal value when the animal was given 50 mg. ascorbic acid per day for 4 days. These two sets of experiments thus give contrary indications as to the ascorbic acid content of the Dael and Biltris sarcoma.

Kellie & Zilva [1936] in the foregoing paper show that the indophenol-reducing material of the JRS can be almost entirely accounted for as ascorbic acid as determined by combined biological and spectrographic tests. Experiments described in this paper show that this is probably also the case in the Dael and Biltris tumour.

It is now well known that the tissues of guinea-pigs subsisting on a scorbutic diet for a few days become depleted of ascorbic acid [De Caro, 1934; Zilva, 1935, 1, 2]. These workers have further shown that the tissues can be rapidly replenished by intravenous injection of ascorbic acid. The injected ascorbic acid is selectively absorbed by those tissues which normally are able to reduce indophenol preferentially, viz. anterior pituitary, adrenal, intestine and liver. The experiments described below show that tumour tissue is also capable of storing and absorbing ascorbic acid selectively.

Table I. *The apparent ascorbic acid content of guinea-pig tissues under different conditions.*

	Live wt. g.	Indophenol-reducing substances expressed as mg. ascorbic acid per g. tissue				
		Dael and Biltris tumour	Liver	Small intestine	Muscle	Carcass
(1) On normal diet	480	A 0.12 B 0.14	0.27	0.31	0.040	0.045
(2) On normal diet	375	0.15	0.25	0.20	—	—
(3) On normal diet	605	A 0.16 B 0.19	0.23	0.27	0.035	0.063
(4) After 8 days' scorbutic diet	410	A 0.025 B 0.030	0.050	0.040	0.020	0.040
(5) After 9 days' scorbutic diet	465	0.023	0.030	0.035	—	0.042
(6) After 10 days' scorbutic diet	690	0.023	0.039	0.034	0.014	0.016
(7) After 10 days' scorbutic diet	340	A 0.017 B 0.010	0.028	0.038	—	—
(8) 50 mg. ascorbic acid injected after 7 days' scorbutic diet. Killed 21 hours after injection	385	C* 0.025 A 0.10	0.20	0.31	0.018	0.022
(9) 50 mg. ascorbic acid injected after 8 days' scorbutic diet. Killed 22 hours after injection	610	A 0.062 B 0.070	0.21	0.21	0.032	0.030
(10) 50 mg. ascorbic acid injected after 8 days' scorbutic diet. Killed 24 hours after injection	385	0.092	0.26	0.21	0.040	0.060
(11) 50 mg. ascorbic acid injected after 9 days' scorbutic diet. Killed 23 hours after injection	395	0.088	0.19	0.21	—	—
(12) 50 mg. ascorbic acid injected after 10 days' scorbutic diet. Killed 24 hours after injection	590	C* 0.009 A 0.12 B 0.11	0.25	0.44	—	—
(13) 50 mg. ascorbic acid injected after 10 days' scorbutic diet. Killed 22 hours after injection	475	A 0.12 B 0.15	0.23	0.27	0.021	0.052
(14) 50 mg. ascorbic acid injected after 10 days' scorbutic diet. Killed 22 hours after injection	725	A 0.11 B 0.13	0.60	0.40	0.034	0.048
(15) 50 mg. ascorbic acid injected after 11 days' scorbutic diet. Killed 24 hours after injection	495	A 0.08 B 0.13	0.20	0.31	0.027	0.032
(16) 50 mg. ascorbic acid injected after 11 days' scorbutic diet. Killed 22 hours after injection	610	0.15	0.24	0.27	0.027	0.045
Average of animals on normal diet	—	0.15	0.25	0.26	0.038	0.054
Average of animals on scorbutic diet	—	0.018	0.035	0.035	0.017	0.033
Average of animals on scorbutic diet after injection of ascorbic acid	—	0.11	0.27	0.29	0.028	0.041
(Average dry weight of tissue as % wet weight	—	16.5	28.3	14.5	24.0	—)
Average ascorbic acid content on normal diet calculated on dry weight	—	0.091	0.88	1.65	0.16	—
Average ascorbic acid content on scorbutic diet calculated on dry weight	—	0.11	0.12	0.24	0.071	—
Average ascorbic acid content on scorbutic diet after injection of ascorbic acid calculated on dry weight	—	0.67	0.96	2.00	0.12	—

A, B, and C refer to different tumours in the same animal. * C was removed before injection of ascorbic acid.

EXPERIMENTAL.

Guinea-pigs were grafted with the Dael and Biltris sarcoma (for the strain of which we are indebted to Dr Watson of the Imperial Cancer Research Fund). One month after the tumour had been grafted the animals were placed on the scorbutic diet described by Bracewell *et al.* [1930] for 8–10 days. The animals were then injected in the jugular vein under generalized ether anaesthesia [cf. Zilva, 1935, 2] with 50 mg. ascorbic acid dissolved in 1 ml. H_2O and neutralized immediately before injection. For gifts of ascorbic acid thanks are due to Prof. Szent-Györgyi and the Hoffman La Roche Chemical Works, Ltd. The urine of the animals was collected and ascorbic acid determined as a check on the injection technique. On the day following the injection of ascorbic acid the guinea-pigs were killed and the ascorbic acid content determined by titration of trichloroacetic acid extracts of the tissues with 2:6-dichloroindophenol, standardized against pure ascorbic acid. Other animals were examined after feeding with normal diet including cabbage *ad lib.* and others were killed and examined after having been fed on a scorbutic diet. The dry weight of the guinea-pig tissues was determined and the average ascorbic acid content of the tissues is given as calculated on wet and on dry weight basis (Table I). The average indophenol-reducing value calculated as ascorbic acid of the tumour tissue under conditions of ascorbic acid deficiency is 0.018 mg. per g. which is increased to 0.11 (sixfold increase) on injection of ascorbic acid. This increase is similar to that occurring with liver, 0.035–0.27 (eightfold), and small intestine, 0.035–0.29 (ninefold), and much greater than the increases found with muscle (twofold) or carcass (between two- and three-fold). The results show that tumour tissue selectively absorbs ascorbic acid and thus resembles liver and intestine which have been shown to fix ascorbic acid [Zilva, 1935, 1].

The ascorbic acid content of the tumours of animals which have been injected with ascorbic acid is not quite as high as in the case of animals fed on a normal diet. This is possibly due to the relatively poor blood supply of tumour tissue as compared with liver and intestine.

The extent of the variation of reducing power of tumour extracts with scorbutic diet and ascorbic acid injection makes it seem probable that the indophenol-reducing material of the guinea-pig tumour, as in the case of the rat tumour [Kellie & Zilva, 1936], is mainly ascorbic acid.

When the ascorbic acid content is expressed as a percentage of the dry matter of the tissue instead of the total weight, the ascorbic acid content of the tumour is seen to be of the same order as that of liver but much less than that of the intestine.

SUMMARY.

The indophenol-reducing activity of a guinea-pig sarcoma like that of other tissues is greatly reduced when the animal is kept on a scorbutic diet; injected ascorbic acid is then selectively absorbed by the tumour tissue and those tissues which normally contain ascorbic acid.

Thanks are due to Dr S. S. Zilva for his advice and criticism on these experiments.

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CLXXIV. METABOLISM OF POLYCYCLIC COMPOUNDS.

III. ANTHRYLMECAPTURIC ACID.

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(Received 19 May 1936.)

ALTHOUGH phenylmercapturic acid has not been isolated there are indications that benzene is excreted partly in that form in dogs [Callow & Hele, 1926]. Bourne & Young [1934] have isolated α -naphthylmercapturic acid from the urine of rabbits and Stekol [1935] from the urine of dogs fed on naphthalene. It has been shown that anthracene is excreted as different stereoisomerides of dihydroxydihydroanthracene [Boyland & Levi, 1935] and it was stated at that time that there were indications of anthrylmercapturic acid in the urine of rats and rabbits fed on anthracene. We have now isolated this mercapturic acid and determined its properties and structure.

EXPERIMENTAL.

Rats and rabbits were fed on the diet described in the previous paper [Boyland & Levi, 1935] which contained anthracene. Sulphur compounds in the urine were determined according to Folin [1905] and some results are shown in Table I. The feeding of anthracene to rats appears to cause an increase in the

Table I. *Sulphur content of urines.*

	Number of animals yielding pooled urine	Free SO ₄	Ethereal SO ₄	Organic S	Total
Normal rats mg. BaSO ₄ per 100 ml. urine	10	436	76	232	744
As % total S		58	11	31	—
Rats fed with anthracene mg. BaSO ₄ per 100 ml. urine	20	381	114	301	796
As % total S		48	14	38	—

proportion of organic sulphur excreted. If the increased organic sulphur were due entirely to anthrylmercapturic acid there should be about 1 g. of that substance per litre urine. Actually only 100 mg. have been isolated in a pure condition from each litre of urine. We have isolated a maximum amount of 20 mg. per litre from rabbit urine.

Isolation of anthrylmercapturic acid.

The method used for the isolation of the anthrylmercapturic acid is in part similar to that used by Bourne & Young [1934] for α -naphthylmercapturic acid. The urine after the removal of the dihydroxydihydroanthracene by prolonged

ether extraction as previously described was acidified with HCl and extracted four or five times by shaking with chloroform. Unfortunately a continuous extractor could not be used because the chloroform tended to form stable emulsions with the acidified urine. The united chloroform extracts were filtered and extracted with aqueous NaHCO_3 . The alkaline extract was acidified with HCl and the precipitated crude mercapturic acid was redissolved in chloroform. The chloroform solution was dried, evaporated to small volume and treated with 3-4 vol. of hot benzene. On standing, brown crystals of mercapturic acid separated. This product was purified by means of the sodium salt as described by Bourne & Young for α -naphthylmercapturic acid, using 4N NaOH instead of 2N NaOH. The product was pure enough for most purposes and for analysis was twice recrystallized from a large volume of toluene or from a mixture of benzene and alcohol.

Properties.

The substances isolated from both rat and rabbit urines were similar in appearance and properties, crystallizing in pale yellow needles, soluble in sodium bicarbonate solution, insoluble in cold water, slightly soluble in hot water and soluble in hot concentrated HCl and in 30% toluenesulphonic acid solution, sparingly soluble in benzene, moderately soluble in chloroform and ether and easily soluble in alcohol, acetone and acetic acid. Other properties of the pure products are given in Table II.

Table II.

	M.P. ° C.	$[\alpha]_D^{20}$ in $\text{C}_2\text{H}_5\text{OH}$	$[\alpha]_D^{20}$ in dioxan	Equiv. wt.
Anthrylmercapturic acid from rat urine	195	- 6° (c = 0.8%)	+ 45° (c = 0.8%)	358
Anthrylmercapturic acid from rabbit urine	193	- 10° (c = 1.5%)	+ 46° (c = 1%)	351
Theory	—	—	—	339

Analysis of acid from rat urine.

	C	H	N	S	Acetyl (Roth)
Found					
Weiler	67.7	5.3	3.9	9.0	—
Schoeller	67.4	5.1	3.9	9.2	—
Roth	—	—	—	—	10.5, 10.3
$\text{C}_{19}\text{H}_{15}\text{O}_3\text{NS}$ requires	67.8	5.0	4.1	9.4	12.7

The figures for the acetyl content are too low but the compound was extremely difficult to hydrolyse and the acetyl content difficult to determine. Attempts to isolate the deacetylated compound have so far been unsuccessful, as the mercapturic acid resisted boiling 10% HCl or 30% toluenesulphonic acid for 24 hours. On heating in a sealed tube with 10% HCl at 130° decomposition with formation of H_2S , anthracene and other products occurred. The acetyl determinations were made by hydrolysis with alcoholic KOH which would cause breakdown to thioanthrol; they do, however, indicate that the molecule contains an acetyl group.

Alkaline hydrolysis.

Hydrolysis with boiling 2N NaOH caused liberation of ammonia which continued for several hours. The solution was then allowed to oxidize in air and a little of a previously undescribed dianthryldisulphide was precipitated.

When 0.5 g. mercapturic acid was boiled with 4N NaOH for 10 min. a yellow precipitate was formed. The mercaptan and disulphide were extracted from the cooled hydrolysis mixture with ether after acidifying. The ether was evaporated and the residue oxidized in cold acetic acid with H_2O_2 . On addition of water the

dianthryldisulphide was precipitated, yield 0.25 g. After crystallization from glacial acetic acid it formed pale yellow prisms, m.p. 191–193°. It was soluble in benzene, ether and acetic acid but only slightly soluble in C_2H_5OH .

Analysis. (Found (Schoeller): C, 80.0; H, 4.48; S, 15.2%. $C_{28}H_{18}S_2$ requires C, 80.4; H, 4.3; S, 15.3%.)

The structure of the dianthryldisulphide.

The dianthryldisulphide could not be the 9:9-dianthryldisulphide, which is an orange-yellow compound melting at 223° [Cooke *et al.* 1925] which on oxidation yields anthraquinone.

The position of the sulphur atom in the molecule was determined by oxidation of the disulphide with boiling HNO_3 to anthraquinonesulphonic acid. After driving off the excess HNO_3 the anthraquinonesulphonic acid was treated with boiling HCl and $KClO_3$ when a chloroanthraquinone separated. The chloroanthraquinone was recrystallized from C_2H_5OH and melted at 158°.

(Found: (Schoeller) Cl, 14.4%. $C_{14}H_7O_2Cl$ requires Cl, 14.7%.)

The substance was identified (m.p. and mixed m.p.) with an authentic specimen of α -chloroanthraquinone, whence it follows that the disulphide must be α -dianthryldisulphide and the product isolated from urine is probably 1-anthrylmercapturic acid.

In addition to the metabolic products of anthracene which we have described, the urine of rats and rabbits given food containing anthracene contains a labile compound which readily yields crystalline anthracene on boiling with acid. This "anthracenogen" is probably a derivative of hydroxydihydroanthracene.

From the $CHCl_3$ extract of urine from rats and rabbits fed with anthracene a small amount of 9:10-anthraquinone has been isolated, which is possibly a contaminant of anthracene present in the animals' food and not a true metabolic product.

The probable metabolic products of anthracene are therefore (1) 1:2-dihydroxy-1:2-dihydroanthracene, (2) the glycuronic acid of (1), (3) a hydroxydihydroanthracene, (4) 1-anthrylmercapturic acid, (5) possibly 9:10-anthraquinone.

SUMMARY.

The same 1-anthrylmercapturic acid has been isolated from the urines of rats and rabbits fed on a diet containing anthracene. Rats produce more of the mercapturic acid than do rabbits under the same conditions.

One of us (A. A. L.) has pleasure in thanking the Sir Halley Stewart Trust for a Fellowship held during the progress of this work.

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CLXXV. CHANGES IN THE ASCORBIC ACID AND GLUTATHIONE CONTENTS OF STORED AND SPROUTING POTATOES.

By LIONEL BRADLEY PETT.¹

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(Received 29 May 1936.)

THE indication given by Guthrie [1932] that the glutathione content of potatoes increases on sprouting has been investigated. Ascorbic acid has been included in the study and the exact time relations of this change, as well as the effects of storing the potatoes at different temperatures are here reported.

The ascorbic acid and glutathione contents of potatoes seem to be at a maximum just at maturity and to decline gradually on storing even when the potatoes are refrigerated, tending to come to a certain low plateau value. Forced or natural sprouting at any point in this decline, or plateau, resulted in a marked rise to rather high values, followed by a rapid drop to the low level again. Remarkable similarity between the changes in glutathione and in ascorbic acid has been observed.

The observed instability of ascorbic acid (vitamin C) on storage is of the greatest importance from the viewpoint of the nutritive value of potatoes.

EXPERIMENTAL.

Storage. Newly lifted potatoes were scrubbed, dried and stored at 15°, 10° or 5°. The author is grateful to the Director of the Low Temperature Research Station, Cambridge, for the opportunity of using their facilities and to Dr J. Barker, of the Station, for his advice and interest. Early potatoes were Duke of York variety, the late were at first Great Scot, later King Edward.

Sprouting. Moist sand in flat pans at 15–16° was used. The potatoes were usually cut in two. Late potatoes, which have a non-sprouting period of about 6 weeks after digging, were forced to sprout by treatment with ethylene chlorohydrin [v. Guthrie, 1932].

Occasional moisture determinations varied from 75.2 to 73.3% during the 5–8 days of sprouting. Results are not corrected for moisture except in the case of potatoes which had been stored 3 months.

Extraction. The potatoes, usually 4–6 in number, were scrubbed, cut into cubes of about 1 cm. dimensions, mixed and sampled by quartering until a quantity of about 50 g. remained. 25 g. were at once weighed into a beaker and just covered with 10% trichloroacetic acid, or 3% salicylsulphonic acid, (which has a slight stabilizing effect on the ascorbic acid). As soon as convenient the cubes were put in a mortar, smashed and ground to a pulp with sand. The pulp was quantitatively transferred to a graduated cylinder, rinsing with the acid from the beaker and the whole made up to a definite volume, usually 40 ml. Frequent vigorous shaking for an hour, followed by suction filtration yielded a slightly opalescent filtrate of about 30 ml. This filtrate contained 96–98% of the glutathione, and 85–92% of the ascorbic acid and, in general, was used directly

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or the present work. A second extraction of the residue in 10–15 ml. trichloroacetic acid yielded most of the remaining ascorbic acid. The procedure of Guthrie & Wilcoxon [1932] of dropping cubes into boiling water was found to give seriously lower values for both GSH and ascorbic acid, indicating destruction. The increased ascorbic acid values found by McHenry & Graham [1935] were possibly due to difficult end-points with a cloudy solution and adsorption of dye on starch.

Estimation of ascorbic acid. An aliquot of the extract after reduction and adjustment to ca. pH 2, was titrated with 2:6-dichlorophenolindophenol of about 0.004 % strength (the Tillmans titration). The dye was made up in *M*/500 phosphate buffer at pH 7.0, and stored at 0°. After the original standardization with pure ascorbic acid it was tested daily with a solution of *M*/10 ferrous ammonium sulphate, dissolved in *M*/500 H₂SO₄, covered with paraffin oil and set up permanently connected to the burette. The dye solution remained fairly constant for 3 weeks or more.

The dye was usually used in the burette. With small amounts of ascorbic acid in the test solution it is convenient to make a standard solution of pure ascorbic acid, add to the extract a given volume (an excess) of dye, and immediately back-titrate with standard ascorbic acid. By this procedure only quick-reacting substances are determined, and this makes the method more specific, since ascorbic acid reduces the dye much faster than other substances liable to be present, such as cysteine and glutathione [Hopkins *et al.*, 1935]. The results are expressed as mg. ascorbic acid per 100 g. fresh tissue, recognizing the possibility that not ascorbic acid alone was determined. The procedure of preliminary precipitation with mercuric acetate, recommended by Van Eckelen & Emmerie [1936], invariably gave results higher than the above and not as uniform. Recently Wachholder & Podesta [1936] have suggested that the Tillmans titration should be discarded as too unspecific. Their criticism is based on comparison with colorimetric methods, which are themselves not of proved specificity and are considered better for the simple reason that they give lower values.

Estimation of glutathione. The extract, or a portion of it, was treated with powdered zinc, shaken a few minutes and filtered. An aliquot, usually 10 ml., received 2 drops of 1 % starch, 2 ml. of 5 % KI and sometimes 1 ml. of *N* H₂SO₄. It was titrated with KIO₃ of suitable strength, made by diluting (usually 1 : 10) a stock solution containing 0.214 g. per 100 ml. The latter was standardized against cysteine or glutathione.

The figure thus obtained was presumed to represent the combined ascorbic acid and glutathione contents and was corrected for ascorbic acid. The results, calculated as mg. GSH per 100 g. fresh tissue, thus represent the total iodine titration less a correction. They are called glutathione with the reservation that the method greatly lacks specificity.

RESULTS.

1. Preparation of glutathione from sprouted potatoes.

The results of Guthrie [1932] in preparing crystalline glutathione from sprouted potatoes have been confirmed, using about the same procedure. As pointed out by Guthrie this is the only known case of actual isolation of glutathione from a plant tissue. The white crystalline material obtained, which gave a strong nitroprusside reaction, contained 10.0 % S, but was not subjected to further analysis.

2. Effect of sprouting fresh stock potatoes.

An increase in glutathione during germination has been mentioned chiefly by Guthrie [1932, 1933]. An extensive literature, however, is available relating to vitamin C and germination. No previous study of potatoes has been found.

Fig. 1 shows that a large and rapid rise occurs in the glutathione and ascorbic acid contents of sprouting potatoes, reaching a maximum at 2 days, followed by

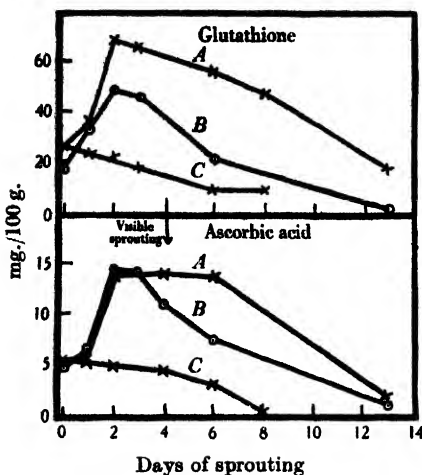


Fig. 1.

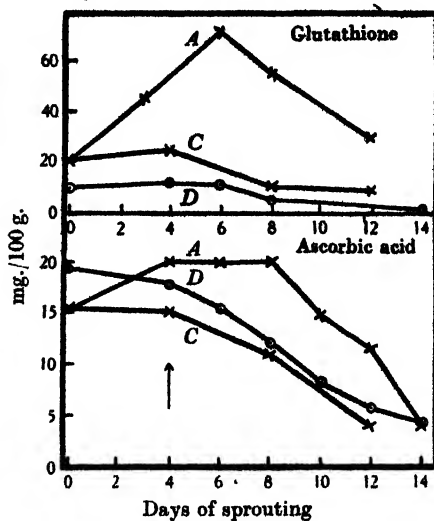


Fig. 2.

Fig. 1. Changes in the glutathione and ascorbic acid of potatoes during sprouting. A, Late (treated to sprout); B, early (sprouting); C, late (not sprouting).

Fig. 2. Changes in the glutathione and ascorbic acid of potatoes during sprouting. A, Late (treated to sprout); C, late (not sprouting); D, early (not sprouting).

a gradual decline to a very low value after 13 days. If no sprouting occurred no rise occurred. The same general effect has been observed many times but the exact time relations and the shapes of the curves may vary considerably. This is shown by Fig. 2.

3. Effects of sprouting stock which had been stored.

This stock had been stored 3 months at 5° and had dehydrated somewhat, containing 56% moisture. The moisture content varied during sprouting and the results were calculated on the dry weight. The usual large increases in GSH and ascorbic acid were found even when calculated on fresh weights. The difference between cut and uncut stock was here more pronounced than usual, but in all previous cases some lag of the uncut had been observed. It is, of course, known that in cut potatoes respiration is greatly increased and possibly some metabolic processes are hastened with which the above substances are concerned.

4. Effect of storing potatoes at 5°, 10° and 15°.

The original plan of the investigation did not include a complete study of this aspect of the problem, and systematic results are not available. A more complete study, including animal tests, is planned for the near future.

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The effect of glutathione seems not to have been investigated before. On the other hand, the stability of ascorbic acid has been extensively studied in many tissues, though notably seldom in potatoes.

McKittrick & Thiessen [1932] reported some loss of vitamin C in potatoes after storing 6 months, but exact figures are not given. Yanovska [1933], to protect guinea-pigs from scurvy, found that 4 times more of stored potatoes were needed than of fresh. Murri *et al.* [1934] found that cranberries at 0° and 8° lost some of their vitamin C, apples at 10° lost all, whilst potatoes lost none. Recently Woods [1935] reported no loss of vitamin C after 3 months' storage. In this case the initial rapid drop may have been over at the start of the experiment.

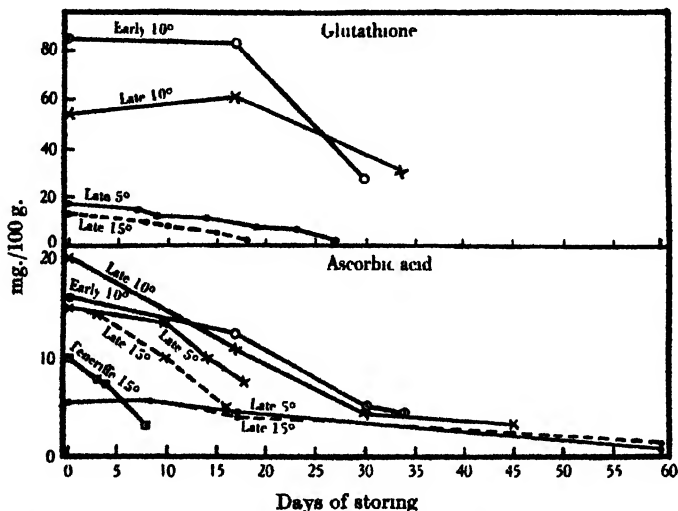


Fig. 3. Changes in the glutathione and ascorbic acid contents of different stocks of potatoes stored at 5°, 10° and 15°.

Fig. 3 shows the relation between time of storing at 5°, 10° and 15° and the contents of ascorbic acid and GSH, using different stocks of potatoes. The loss was rapid at all temperatures, though slightly more at 15°, the rate depending on the initial value. The ascorbic acid shows a tendency to come to a certain level, after which a much slower decrease occurs. In Fig. 3 the potatoes at 5° were stock purchased late in the season and had presumably reached the level obtained in 30 days by the other stock. The results also suggest a variation in different varieties of potatoes and possibly too in the same variety grown on different soils, but this has not been thoroughly studied. Such variations were suggested for vitamin C in potatoes by Richardson *et al.* [1931].

DISCUSSION.

The results have a practical significance in their bearing on nutrition. The importance of vitamin C in diet needs no discussion, and the suggestion has been made that potatoes are the chief source of this vitamin for poor families. If present methods of storing threaten even this source, as suggested by these results, other methods should be explored. The results on sprouting potatoes indicate a possible solution of the problem, since a very short period of sprouting

(2-4 days), which was not sufficient to show any change in the "eyes" of the potatoes, restored a good part of the vitamin. On the other hand, once visible sprouting begins the ascorbic acid content falls rapidly to the vanishing point.

The results have also a theoretical importance in clearly demonstrating for the first time the great increase in glutathione during the sprouting of potatoes. A similar increase in germinating wheat seeds has since been observed and will be reported elsewhere. At the present time it is difficult to suggest specific reasons or functions associated with these changes. The increase is made before real sprouts appear and appears to be generalized throughout the tuber. The ascorbic acid content of the sprouts after about 2 weeks is higher than that of the tuber tissue, but the glutathione content is lower, this being the only point at which the usual parallelism between these two fails.

It is, indeed, of especial interest that the ascorbic acid and glutathione contents undergo the same changes, rising and falling together, in view of the intimate relationships found by Bersin *et al.* [1935] in animal tissue and by Hopkins (unpublished). It seems unlikely that a chemical reaction between the two is involved, but since the presence of GSH protects ascorbic acid from reversible oxidation (Hopkins), and since the total amounts of the two change simultaneously, it is possible that they are linked in action by being thermodynamically suitable to each other, with redox potentials in advantageous relation.

SUMMARY.

1. Sprouting of potatoes is associated with a large rise in the content of ascorbic acid (vitamin C) and of glutathione, followed by a decrease. Time curves for these changes are given. This occurs even after storing potatoes some months at 5°.
2. Potatoes stored at 15°, 10° or 5° rapidly lose ascorbic acid and glutathione, reaching a level at 20-30 days after which the loss is much slower. The importance of this loss from a nutritive standpoint is discussed.
3. Guthrie's isolation of crystalline glutathione from sprouted potatoes has been repeated.

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Note added 4 July 1936. Olliver [*J. Soc. chem. Ind.* 1936, **55**, 153], in an investigation of cooking and storing effects, found a rapid decrease in the ascorbic acid of stored potatoes, thus confirming the above results.

I. A. R. I. 75.

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